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Elucidation of mechanism of disease resistance and persistence in chronic myeloid leukemia

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Ashu Kumari

aus Allahabad, India

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Dekan: Prof. Dr. med. Matthias Rothmund

Referent: Prof. Dr. Andreas Burchert

Korreferent: Prof. Dr. Michael Lohoff

I dedicate my thesis to my parents

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SUMMARY

Chronic myeloid leukemia (CML) is a clonal disorder of the hematopoietic stem cell caused by the BCR-ABL receptor tyrosine kinase. Imatinib mesylate (IM) is an inhibitor of BCR-ABL and has been approved for the treatment of CML. IM is well tolerated and highly efficacious as it induces stable long-term remissions in the vast majority of patients. Despite its efficacy, a still unresolved issue associated with IM therapy is IM resistance in progressed phases of CML and long-term disease persistence. It has been shown that BCR-ABL mRNA and BCR-ABL-positive progenitor and stem cells remain detectable after years of therapy. Based on the BCR-ABL expression analysis and short term IM exposure experiments of IM-naïve, first diagnosis CML precursor cells it has been suggested that BCR-ABL over-expression contributes to a major extend to the incapability of IM to kill and eradicate primitive precursors and CML stem cells. Interestingly, the BCR-ABL expression level in actual persisting CML precursor clones, and the impact of long term IM therapy on the eradication of CML precursors from different bone marrow compartments was never been thoroughly investigated.

Here we studied a putative novel IM persistence mechanism by directly investigating in residual BCR-ABL-positive progenitor and stem cell clones in chronic phase CML patients in major molecular remission (MMR) under IM. We could first show that IM not only eliminates BCR-ABL positive cells from both primitive (stem cell containing) and more mature bone marrow precursor compartments but also that, in contrast to the currently proposed model, persisting primitive and mature BCR-ABL positive colony forming clones (CFU) expressed significantly less BCR-ABL than CML CFU isolated from initial diagnosis patients. Indeed, lower BCR-ABL expression reduces IM sensitivity of primary bone marrow progenitors engineered to express BCR-ABL. Whereas high BCR-ABL expression level increased IM responsiveness but also the frequency of BCR-ABL kinase mutation development as the most important IM resistance

mechanism. This would explain the low propensity of secondary IM resistance in patients, which do achieve a good molecular remission with IM.

Another regulator of BCR-ABL kinase point mutation development has been identified with ICSBP, an interferon regulated gene that was previously found by our group to be downregulated in CML. Lack of ICSBP expression in murine myeloid 32D-BA cells conferred BCR-ABL independent IM resistance and limits the development kinase point mutations. Together, here we described two novel and clinically relevant mechanisms of CML persistence and resistance under IM, which may provide a novel perspective for reassessing treatment strategies aiming at eradicating residual disease in CML and to overcome IM resistance.

ZUSAMMENFASSUNG

Die chronische myeloische Leukämie basiert auf einer klonalen Transformation hämatopoetischer Stammzellen, für die die Translokation t(9;22)(q34;q11) mit Entstehung der konstitutiv aktivierten BCR-ABL Tyrosinkinase kausal verantwortlich ist.

IM ist ein spezifischer Inhibitor von BCR-ABL, der speziell für die Behandlung der CML entwickelt wurde und klinisch hochwirksam ist. Die große Mehrheit der mit IM behandelten Patienten erreichen stabile Langzeitremissionen. Trotz dieser enormen Wirksamkeit gibt es weiterhin zwei entscheidende Probleme unter IM. Zum einen kommt es bei einem Teil der Patienten unter IM zur Resistenzausbildung. Andererseits scheint die Substanz zwar antiproliferativ zu wirken, aber nicht in der Lage zu sein, CML Progenitor- und Stammzellen abzutöten. Dies führt zu Persistenz der Erkrankung im Knochenmark von CML Patienten und macht eine IM Dauertherapie als Standard erforderlich. Basierend auf in vitro Untersuchungen wurde eine Überexpression von BCR-ABL in CML Stammzellen unbehandelter CML Patienten als ein wesentlicher Mechanismus für IMpersistenz proklamiert, obwohl die BCR-ABL Expressionshöhen persistierender CML Vorläuferzellen unter IM unbekannt sind. Ebenso unklar ist, ob IM in vivo in der Tat nicht in der Lage ist, CML Stammzellen abzutöten.

Im Rahmen dieser Doktorarbeit wurde ein neuartiger IMpersistenzmechanismus beschrieben. Im Knochenmark von molekular in sehr guter Remission befindlichen Patienten unter IM wurde nach persistierenden CML Klonen gesucht, deren Frequenz und BCR-ABL Expressionshöhen bestimmt und mit denen von CML Patienten bei Erstdiagnose verglichen. Im Gegensatz zum aktuell etablierten Persistenzmodell konnte gezeigt werden, dass IM zu einer deutlichen Abnahme der BCR-ABL positiven Stammzellen bei Patienten in Remission unter IM führt und dass persistierende Klone BCR-ABL nicht überexprimierten, sondern nur sehr geringe BCR-ABL Mengen aufwiesen. Es konnte dann an primären

Knochenmarkzellen gezeigt werden, dass eine niedrige Expression von BCR-ABL in der Tat zu einer IMresistenz führt, währenddessen die BCR-ABL Überexpression eine höhere Empfindlichkeit auf IM induzierte, andererseits aber auch die Entstehung von BCR-ABL Kinasemutationen als einem wesentlichen Mechanismus von IM resistenz katalysierte. Dies würde erklären, warum Patienten mit guter molekularer Remission unter IM eine vernachlässigbare Rate an sekundär IMresistenzausbildung aufweisen.

Etwa 80% der CML Patienten zeigen eine reduzierte Expression des interferonregulierten Faktors, ICSBP. Die fehlende Expression von ICSBP wurde hier als ein BCR-ABL unabhängiger Mediator von IMresistenz und Kinasemutationsentstehung identifiziert.

Diese Arbeit beschreibt somit zwei neue und potentiell klinisch relevante Ursachen von IM resistenz und –persistenz. Deren Kenntnis könnte zur Re-evaluation von Strategien zur Eradikation residueller CML Stammzellen und dem Überkommen von IM resistenz beitragen.

1. ABBREVIATIONS

All units of measurement are abbreviated according to the International System of units (SI).

A	adenosine
ABL	abelson
AID	activation-induced cytidine deaminase
ALL	acute lymphoblastic leukemia
APS	ammoniumperoxodisulfat
ATP	adenosine triphosphate
BC	blast crisis
BCR	breakpoint cluster region
bp	base pair
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
CML	chronic myeloid leukemia
CMP	common myeloid progenitor
DA	dasatinib
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTPs	2'-deoxynucleoside-5'-triphosphates

DTT	dithiothreitol
EDTA	ethylene diaminetetraacetic acid
EtBr	ethidium bromide
FACS	fluorescence-activated cell sorter
FCS	fetal calf serum
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte/macrophage colony-stimulating factor
GMP	granulocyte macrophage progenitor
HEPES	(2-Hydroxyethyl)-1-piperazineethanesulphonic acid
HRP	horse radish peroxidase
HSC	hematopoietic stem cells
IAD	IRF-association domain
ICSBP	interferon consensus sequence binding protein
IFN	interferon
IRF	interferon regulatory factor
ISRE	IFN stimulated response elem
IL-3	interleukin-3
IL-6	interleukin-6
IM	Imatinib mesylate (IM, Gleevec®)
IMDM	Iscoves Modified Dulbecco's Medium

kb	kilobase pair
kD	kilodalton
Lin^-	lineage negative
MRD	minimal residual disease
MEP	megakaryocyte erythroid progenitor
NaCl	sodium chloride
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Ph+	philadelphia chromosome positive
PI3K	phosphatidylinositol 3-kinase
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
rpm	retation per minute
RT-PCR	reverse transcription PCR
SDS	sodium-dodecyl-sulphate
TAE	tris-acetate-EDTA buffer
TBE	tris-borate-EDTA buffer
TE	tris-EDTA
TKI	tyrosine kinase inhibitor

Tris	tris(hydroxymethyl)-amino-methane
U	unit
wt	wild type

2 INTRODUCTION

2.1 Chronic Myeloid Leukemia (CML)

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder (Baikie et al., 1960). In 1960 Nowell and Hungerford described a shortened chromosome 22 known as Philadelphia chromosome (Ph chromosome) generated from chromosomal translocation. This translocation juxtaposes the *c-abl* gene on chromosome 9 and the *bcr* gene on chromosome 22 as t (9;22)(q34;q11) generates the *BCR-ABL* fusion gene (Figure1) (Nowell and Hungerford, 1960; Rowley, 1973). CML was the first human disease to be associated with a consistent cytogenetic abnormality, BCR-ABL, as well as the first cancer to be treated with molecular targeted therapy. (Daley et al., 1990; Fialkow et al., 1977; Heisterkamp et al., 1990; Heisterkamp et al., 1985; Konopka et al., 1985; Nowell and Hungerford, 1960)

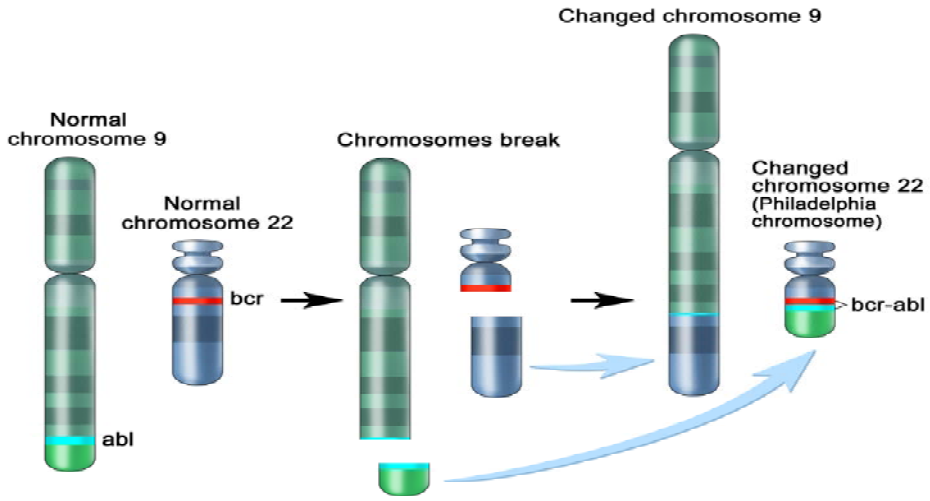

Figure 1: The Philadelphia chromosome translocation. The normal chromosomes 9 and 22 carry the *c-ABL* and *c-BCR* genes, respectively. The translocation results in the formation of a shortened chromosome 22 (the Philadelphia chromosome) carrying the *BCR–ABL* fusion gene.

2.1.1 Clinical characterization of CML

The course of the disease is characteristically triphasic: a chronic phase (CP) lasting three to six years is followed by transformation to an accelerated phase (AP) and then a terminal blast phase (BP) of short duration (Faderl et al., 1999). Chronic phase is characterized by an expansion of immature and mature myeloid cells and retention of hematopoietic differentiation (Lichtman MA., 1995). Patients initially are asymptomatic but also show fatigue, splenomegaly, anemia and high white blood cell counts in the peripheral blood. Both accelerated and blast phases are characterized by a severe reduction in cellular differentiation, with a replacement of mature cells by immature blasts (Lichtman MA., 1995) at this point patients have more severe clinical symptoms including those related to infectious and bleeding complications. Blast crisis essentially resembles acute leukemia which can either be of lymphatic (30%), or more frequently of myeloid character (approx. 70%).

2.1.2 Structure of the BCR and ABL

The N-terminus of BCR-ABL kinase contains the "Cap" region, which is present in 2 different isoforms generated by alternative splicing of the first exon, termed 1a and 1b. ABL 1b contains a C_{14} myristoyl moiety (myristoylation is a process that attaches the fourteen-carbon saturated fatty acid myristate to the amino-terminal glycine of proteins) covalently linked to the N terminus. ABL also contains a tyrosine kinase domain preceded by highly conserved Src-homology-2 (SH2) and SH3 domains (Hantschel and Superti-Furga, 2004). The last exon region contains 4 proline-rich SH3 motifs that function as binding sites for adaptor proteins such as Crk, GRB2 (growth-factor-receptor-bound 2), and Nck, (Feller et al., 1994; Smith et al., 1999) a DNA-binding domain, an actin-binding domain, 3 nuclear localization signals (NLS), and 1 nuclear export signal (NES), which determines ABL subcellular localization in response to environmental stimuli

(Quintas-Cardama and Cortes, 2009). The carboxy terminal contains DNA as well as G and F actin binding domains.

BCR contains a coiled-coil dimerization domain (DD), a serine/threonine kinase domain, a Dbl/CDC24 guanine-nucleotide exchange factor (RHO-GEF) homology domain, a pleckstrin homology domain (PH), a putative calcium-dependent lipid binding site (CaLB), and a Ras-related C3 botulinum toxin substrate (RAC) guanosine triphosphatase–activating protein domain (Rac-GAP). Tyr177 at BCR serves as docking site for GRB2, GRB10 and the ABL proteins through its SH2 domain (Ren, 2005).

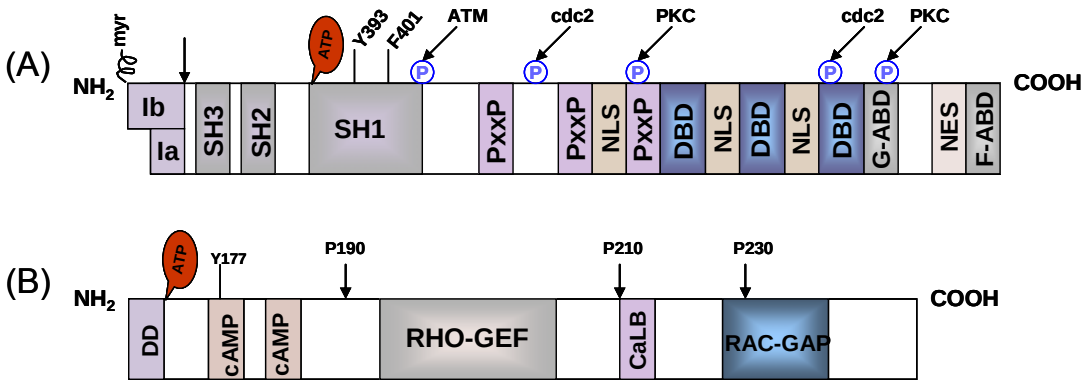

Figure 2: Structure of BCR and ABL protein. (A) Type 1b isoform of ABL protein is slightly longer than 1a because of myristoylation (myr) site for attachment to the plasma membrane. 3 SRC-homology (SH) domains situated toward the NH_2 terminus. The major site of auto-phosphorylation within the kinase domain is Y393. Phenylalanine 401 (F401) is highly conserved in phospho tyrosine kinase (PTKs) containing SH3 domains. Phosphorylation sites by Atm, cdc2, and PKC are shown. The arrowhead indicates the position of the breakpoint in the BCR-ABL fusion protein. (B) At N terminus BCR contains dimerization domain and 2 cyclic adenosine mono phosphate kinase homologous domain. Y177 is the autophosphorylation site crucial for binding to Grb-2.

2.1.3 Molecular anatomy of BCR ABL translocation

The *ABL* gene encodes a ubiquitously expressed, non-receptor tyrosine kinase with a molecular mass of 145 kD ($p145^{ABL}$). The *ABL* gene contains 11 exons among which the first exon has two variants: 1a and 1b, derive from alternative splicing of the first exon (Kurzrock et al., 1988). The breakpoint in the *ABL* gene may occur anywhere over a large 300 kb area at its 5' end, either downstream of exon 1a or upstream of exon 1b, between the two. The *ABL* exons 2 to 11 (also called a2 to a11) are juxtaposed to the 5' part of *BCR*. The major breakpoint cluster region (M-bcr) of the *BCR* gene on chromosome 22 is located between exon 12 and 16 (referred to as b1 to b5) and extends over 5.8 kb.

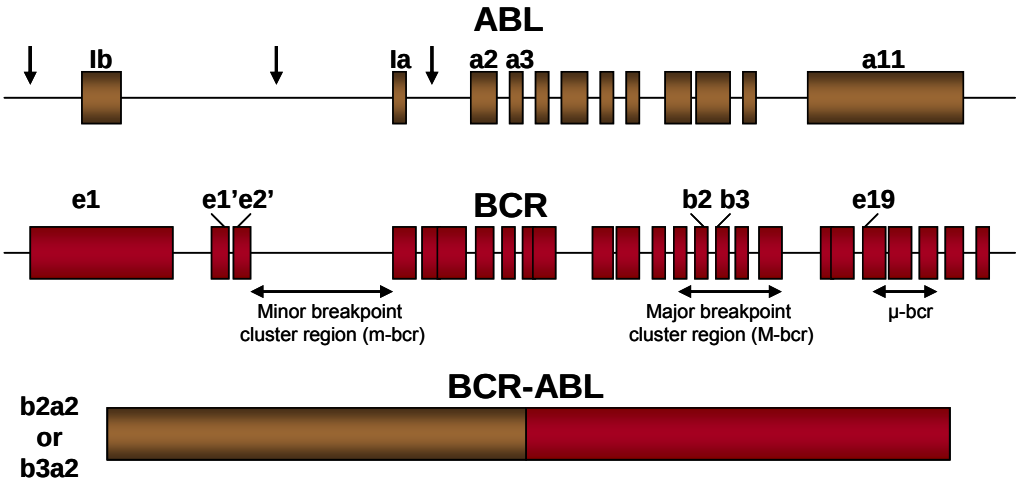

Figure 3: Schematic representation of chimeric *bcr-abl* mRNA generation. The location of breakpoints during translocation of chromosomes 9q and 22q leading to the generation of the Philadelphia chromosome carrying the *BCR-ABL* fusion gene. Exons are shown as boxes (brown for ABL, red for BCR) and breakpoints are indicated by arrows. In most cases of CML, breakpoints occur within the *BCR* gene at 22q11 within a 5.8kb sequence spanning exons b1-b5 known as major breakpoint cluster region (M-bcr). Breakpoints within the *ABL* gene at 9q35 occur in a region of about 30kb at 5' end of the *ABL* gene, either upstream of the first alternative exon 1b, downstream of the second alternative exon 1a or more frequently between the two. All of these possible breakpoints and subsequent chromosomal translocations result in production of an mRNA where *BCR* sequences (exon b2 or b3) are fused to *ABL* exon a2, as a result of the splicing of the primary hybrid transcript leading to generation of different fusion transcripts (b2a2, b3a2).

Two fusion transcripts b2a2 and b3a2 are created, and both translate into a chimeric protein of 210 kD named $p210^{BCR-ABL}$ (Kurzrock et al., 1988) (Figure3).

In 95% of *BCR-ABL* positive CML, the leukemic cells have either b2a2 or b3a2 transcripts, but in 5 percent of cases, alternative splicing events cause the expression of both fusion products (Melo, 1996)

2.2 Signaling pathways of BCR-ABL

BCR-ABL allows multiple protein-protein interactions which involves diverse intracellular signaling pathways. Several domains in BCR-ABL serve to bind

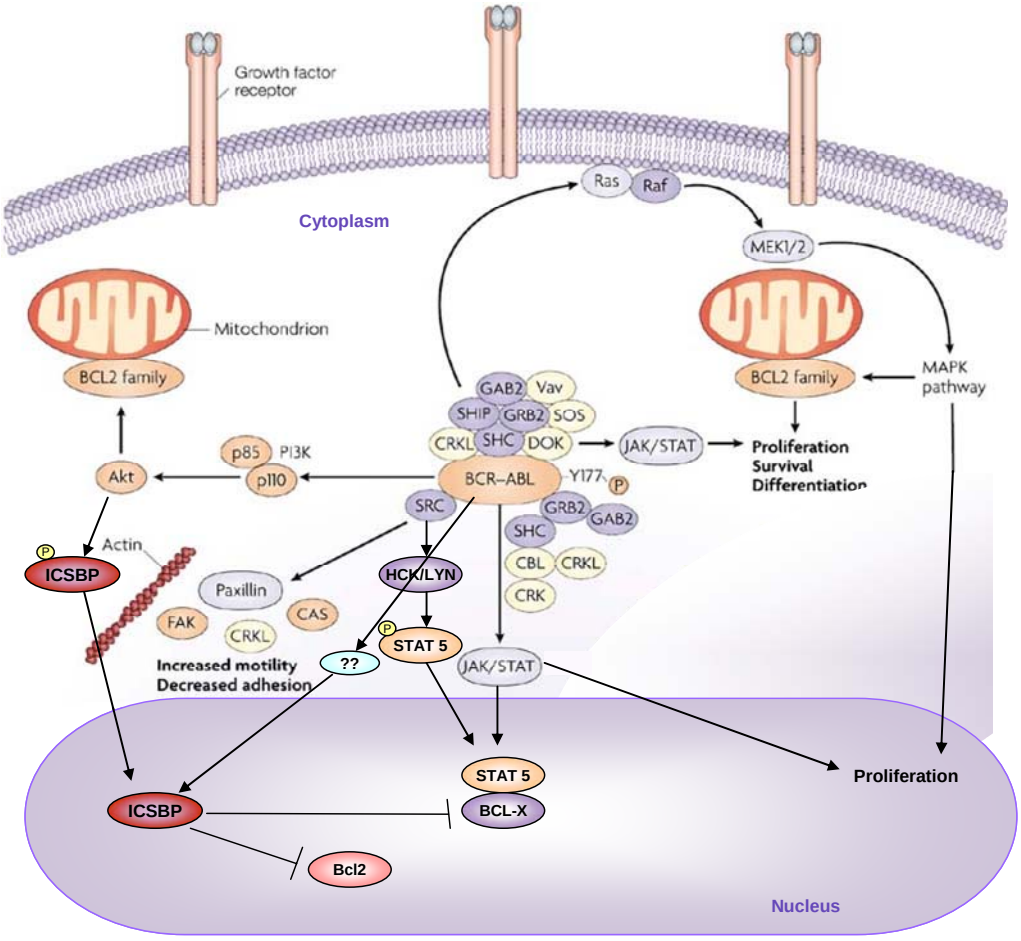

Figure 4: BCR-ABL-dependent signaling pathways. modified from (Weisberg et al., 2007a; Weisberg et al., 2007b)

adapter proteins such as growth factor receptor-bound protein 2 (GRB2), CRK-like protein (CRKL), casitas B-lineage lymphoma pro-oncogene protein (CBL), and SRC homology 2-containing protein (SHC) (Puil et al., 1994). The SH2 domain of GRB2 binds to a conserved tyrosine residue (Y177) of BCR in $p210^{BCR-ABL}$, which links $p210^{BCR-ABL}$ to RAS, a guanosine triphosphate (GTP) – binding protein involved in the regulation of cell proliferation and differentiation and located at the core of the most prominent signaling pathway in the pathogenesis of CML (Sawyers et al., 1995) (Figure 4)

Signaling events downstream of RAS are not well characterized and may involve mitogen-activated protein kinases (MAPKs), such as the JUN kinase (JUK) (Raitano et al., 1995). Activation of the CRKL or SHC protein which bind to the SH2 and SH3 domains of BCR-ABL, respectively, can also initiate signal transduction of the RAS signaling pathway (Oda et al., 1994; Senechal et al., 1996). Signal transducer and activator of transcription 5 (STAT5) of the JAK/STAT5 pathway can be phosphorylated directly by BCR-ABL, independent of the RAS signaling pathway (Chai et al., 1997; Ilaria and Van Etten, 1996), which leads to the up-regulation of the anti-apoptotic protein BCL-XL which is repressed by transcription factor interferon consensus sequence binding protein (ICSBP). BCR-ABL abrogates transcription of ICSBP through an unknown mechanism which releases ICSBP mediated inhibition of *BCL-2* and *BCL-X* gene transcription and results in increased survival of myeloid progenitor (Burchert et al., 2004). Furthermore, phosphatidylinositol-3 kinase (PI-3K) pathway is activated by BCR-ABL, which results in enhanced pro-mitogenic and anti-apoptotic signals (Skorski et al., 1995). C-Myc has also been identified to be involved in the BCR-ABL signaling pathway (Afar et al., 1994).

Although the different signaling pathways of BCR-ABL have been intensively studied, none has been identified to explain all phenotypic features described in CML. However, as an end result, the uncontrolled kinase activity of BCR-ABL gives rise to deregulated cell proliferation, decreased adherence of leukemic cells to the bone marrow stroma, and inhibition of apoptosis.

2.3 Therapy for CML

2.3.1 First generation of Tyrosine kinase inhibitor IM

Initial treatment modalities of CML include cytotoxic chemotherapies such as busulfan and hydroxyurea. Later stem cell transplantation and interferon α therapy have already offered the possibility of complete and durable cytogenetic responses (Talpaz et al., 1991). However, IM was developed as the first molecularly targeted therapy to specifically inhibit the BCR-ABL tyrosine kinase in Philadelphia chromosome positive CML (mechanism of action in figure 5). Because of the excellent hematologic and cytogenetic responses, IM has moved toward first-line treatment for newly diagnosed CML. However, emergence of resistance and persistence of BCR-ABL positive cells (residual disease) after therapy are two major obstacles to IM based therapies for patients with CML.

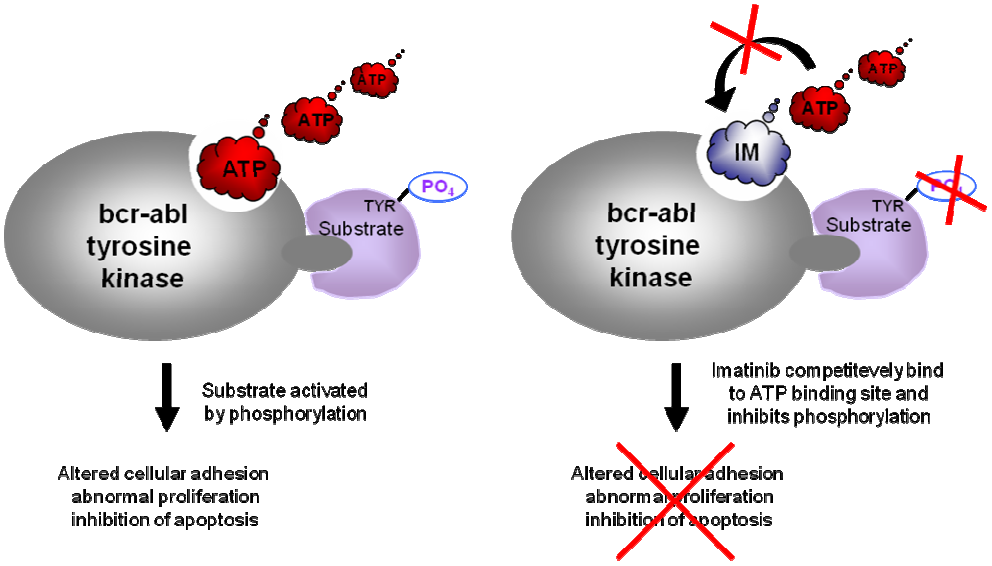

Figure 5: Mechanism of action of IM. The active site of the BCR-ABL tyrosine kinase has an ATP binding site. BCR-ABL phosphorylates its substrate by transfer of the terminal phosphate from ATP to tyrosine residues on its substrates. IM works by binding to the ATP binding site of BCR-ABL and inhibiting the enzyme activity of the protein competitively.

2.3.2 Second generation of tyrosine kinase inhibitor

Dasatinib

Dasatinib inhibits number of kinases, including BCR-ABL, src family, c-Kit, EPHA2 and PDGFRβ (Lombardo et al., 2004; Schittenhelm et al., 2006) Dasatinib is approximately 325 fold more potent against BCR-ABL inhibition (O'Hare et al., 2005) than IM and can bind to both active and inactive conformations of the ABL kinase domain. Dasatinib has been shown to inhibit 18 out of 19 BCR-ABL mutations mediating resistance to IM in-vitro (O'Hare et al., 2005; Schittenhelm et al., 2006; Shah et al., 2006). Dasatinib has activity against many IM resistance-inducing kinase domain mutations of BCR-ABL, including those within the phosphate-binding loop (P-loop) and those within the activation loop and other sites in the COOH-terminal loop (O'Hare et al., 2005; Talpaz et al., 2006; Tokarski et al., 2006). Dasatinib may overcome resistance mechanisms of IM, including alternate signaling pathways involving the Src family kinases and MDR-1 gene overexpression (Thomas et al., 2004). Most of the clinically relevant mutations are inhibited by Dasatinib with the exception of the T315I mutation, which confers resistance to IM, dasatinib, and nilotinib (O'Hare et al., 2005; Tokarski et al., 2006)

Nilotinib

Nilotinib (AMN 107) is another orally available second-generation kinase inhibitor of BCR-ABL, KIT, PDGFR and ephrin receptor kinase. Nilotinib is an analog of IM and it disrupts the ATP-phosphate-binding pocket of the ABL tyrosine kinase and inhibits enzymatic catalytic activity by binding to the inactive conformation of the enzyme, blocking the substrate binding site. Similar to dasatinib, nilotinib has no activity against the T315I mutation but was able to overcome resistance in 32 of 33 IM resistant BCR-ABL mutations.

2.4 Mechanisms of resistance to IM

2.4.1 BCR-ABL independent mechanisms of resistance

Intracellular uptake of IM depends on the net result of between influx and efflux of the drug into the cell. The adenosine triphosphate-binding cassette (ABC) transporter ABCB1 (or MDR-1), for example is a transmembrane protein that mediates multidrug resistance through induction of increased efflux of different chemotherapeutic agents. Overexpression of ABCB1 is also a possible mechanism for resistance to IM, it was first reported by Mahon and colleagues in cells from patients with BP CML (Mahon et al., 2000). Alternatively, inhibition of IM uptake into the cell has been proposed through the human organic cation transporter (hOCT1) and was shown as an important factor regulating intracellular IM availability (Crossman et al., 2005; Thomas et al., 2004).

IM resistance may also result from the acquisition of additional nonrandom cytogenetic aberrancies in Philadelphia positives metaphases, also referred as "clonal evolution". Clonal evolution has been observed more frequently in patients in progressed phase of CML. The most frequent cytogenetic abnormalities associated with clonal evolution are trisomy 8 (34%), isochromosome 17 (20%), and duplicate Ph chromosome (38%) (Johansson et al., 2002) which have been linked to c-*Myc* over expression, loss of 17p, and *BCR-ABL* over expression, respectively (Haferlach et al.).

2.4.2 BCR-ABL Dependent mechanism of Resistance

***BCR-ABL* Overexpression:** Upregulation of the BCR-ABL kinase in association with amplification of the *BCR-ABL* gene was first reported in the Ba/F3 BCR-ABL-r, LAMA84-r, and AR230-r IM-resistant cell lines in the absence of mutations within the *BCR-ABL* kinase domain (Hochhaus et al., 2002; le Coutre et al., 2000). CD 34^+ CML cells expressing high amount of BCR-ABL are much less sensitive to IM, but yield mutant, IM resistant subclones and develop mutations much faster than those with low level of BCR-ABL expression (Barnes et al., 2005).

Point mutation in the kinase domain of BCR-ABL: Point mutations cause amino acid substitutions inside the kinase domain of the BCR-ABL protein and disrupt the binding site of IM on the tyrosine kinase, resulting in a loss of sensitivity to the drug. Mutations can either directly interrupt critical contact points between the drug and the BCR-ABL protein or induce a conformational change, resulting in a protein that is unable to bind IM (Branford et al., 2003b; Hochhaus et al., 2001). There more than 40 point mutations known to occur within the ABL kinase and these may lead to secondary or acquired resistance after IM treatment (Gorre et al., 2001; Weisberg et al., 2006). The first reported mutation mediating resistance was T315I which represents the replacement of a threonine by an isoleucine at amino acid position 315 in the ABL component of the kinase (Gorre et al., 2001). To date, more than 50 different *BCR-ABL* kinase domain mutations have been found to be associated with IM resistance (Branford et al., 2003a; Branford et al., 2002; Shah et al., 2002). BCR-ABL kinase mutations cluster into four main groups. The first group (G250E, Q252H, Y253F and E255K) includes the corresponding amino acids in the nucleotide-binding loop for ATP, also known as the p-loop mutations (Hochhaus et al., 2001; von Bubnoff et al., 2002). The second group of mutations is localized in the IM-binding site and directly interacts with the drug *via* a hydrogen bond (T315I) and Van der Waals' interactions (V289A, T315I and F317L) (Branford et al., 2002; Gorre et al., 2001). The third group of mutations (M388L and H396P) is found in the activation loop (A-loop) (von Bubnoff et al., 2002). These mutations result in a preferred transition of the protein from an inactive conformation to an active conformation to which IM can not bind (Schindler et al., 2000). The fourth group includes amino acids distant from the IM binding site (Branford et al., 2002; Shah et al., 2002), which form a hydrophobic patch between helices E, F and I in the C-terminal lobe of the enzyme, highly conserved region within the tyrosine kinase family (Hubbard, 1997). Despite their different locations, some mutations, such as T315I are completely or near completely insensitive to IM and the second-generation TKIs dasatinib, nilotinib, and bosutinib at clinically achievable doses.

2.5 Leukemic stem cell model

Most leukemia types initially respond well to therapy with partial or even complete remission. However, after a period of minimal residual disease many patients succumb to refractory relapses of the disease. According to stem cell model of leukemia relapse of the disease is due to the selective continued survival of a small, but distinct population of therapy-resistant tumor-initiating cells, commonly referred to as leukemic stem cells (LSC) or CML stem cell (CSC) (Bonnet and Dick, 1997; Lapidot et al., 1994). Interestingly, these CSC, which initially coexist with normal HSCs, have a selective disadvantage in reconstituting NOD-scid mice transplanted with bone marrow or peripheral blood from patients with early phase CML (El-Ouriaghli et al., 2003; Sirard et al., 1996; Verstegen et al., 1999; Wang et al., 1998). Progression to BP CML requires either the expansion of LSC or the acquisition of self renewal property by a subset of committed progenitors. In a mouse model, expression of the hMRP8 $p210^{BCR-ABL}$ transgene targeted to GMP and their myelomonocytic progeny, but not to HSC, renders a phenotype that resembles human CML, including progression to AP and BP (Jaiswal et al., 2003). These data suggest that BP may result from the progressive acquisition of genetic alteration within progenitor downstream of CSC that acquires self renewal or "stemness" property. A comparison of the gene signatures of chronic accelerated, and blast phases suggest that the progression of CML is a two-step process, which includes deregulation of the WNT/β-catenin pathway, the decreased expression of Jun B and Fos, alternative kinase deregulation, such as Arg (Abl2), and an increased expression of PRAME (Radich et al., 2006). Thus hypothetically, CML could be considered as stem cell derived but progenitor driven disorder.

LSC absolutely require self-renewal capability to propagate the disease. Wnt signaling pathway plays an important role in regulating stem cell self-renewal (Taipale and Beachy, 2001). Stabilized β-catenin (a downstream activator of the

Wnt signaling pathway) has been shown to promote the self-renewal of stem cells and other types of progenitor cells (Reya et al., 2001; Zhu and Watt, 1999). Mutations in other signaling pathways that promote progenitor self-renewal, such as Notch and Shh, also contribute to unregulated self-renewal of LSC (Bhardwaj et al., 2001; Varnum-Finney et al., 2000).

2.6 Disease persistence

IM has become the standard front-line therapy for CML by inhibiting BCR-ABL positive leukemic cells. However, despite long-term IM therapy, BCR-ABL positive cells remain detectable during complete cytogenetic remissions (CCR) (Druker et al., 2006; Hughes et al., 2003). Minimal residual disease is almost always detectable and known as disease persistence (Bhatia et al., 2003).

Further studies have demonstrated that IM effectively eradicates Bcr-Abl-positive progenitor cells, but does not target Bcr-Abl-positive $CD34^+$ LSCs (Graham et al., 2002; Jiang et al., 2007). While new inhibitors (nilotinib and dasatinib) that target primary IM-resistant Abl mutants provide treatment options for relapsed patients or patients in blast-crisis CML, these Abl inhibitors also do not target the LSCs in CML. CML persistence supposedly results from an inherent insensitivity to IM of the CML stem and progenitor cells (Copland et al., 2006; Graham et al., 2002; Jorgensen et al., 2007). Stem cell persistence is also supported by clinical evidence showing that IM discontinuation at the time of stable complete molecular remission (CMR) may still result in hematological relapse (Breccia et al., 2006; Cortes et al., 2004; Guastafierro et al., 2009; Rousselot et al., 2007). Various mechanisms may account for CML persistence under IM. These include, for example, BCR-ABL overexpression (Copland et al., 2006; Jiang et al., 2007a; Jiang et al., 2007b), drug in- and efflux mechanisms (Brendel et al., 2007; Engler et al., ; Thomas et al., 2004), and Abl-kinase point mutations (Chu et al., 2005). On the other hand, the number of patients with undetectable BCR-ABL transcripts rises with longer IM treatment duration, and approached 52% after 5 years in a

sub-cohort of the IRIS study (Branford et al., 2007). These clinical results are clearly intriguing in view of the current model that CML precursors and HSC are essentially IM-insensitive.

2.7 Interferon consensus sequence binding protein (ICSBP)

Interferon consensus sequences binding protein (ICSBP), also known as IFN regulatory factor-8 (IRF-8) is a transcription factor of Interferon regulatory factor (IRF) family. IRF family consists of nine cellular transcription factors which share a high homology within the N-terminal at first 115 amino acids. This part comprises the DNA-binding domain (DBD) and therefore binds to the similar DNA element known as interferon stimulated response element (ISRE). ISRE contains the IRF recognition sequence (IRS) AANNGAAA, to which IRF family binds. These IRFs mediate several biological activities such as anti-viral immune response, proliferation, and hematopoietic differentiation (Cohen et al., 2000). ICSBP is constitutively expressed in B cells and monocyte cells, and, at low levels, in resting T cells. Its expression can be further induced by IFN-γ, by combined exposure of macrophages to IFN- γ and lipopolysaccharide (LPS) and by antigenic stimulation of T cells (Barber et al., 1995; Driggers et al., 1990; Nelson et al., 1996; Nelson et al., 1993; Politis et al., 1994; Weisz et al., 1994; Weisz et al., 1992). IFN- alpha also can induce *ICSBP* gene expression in vivo (Schmidt et al., 1998). ICSBP can selectively suppress the expression of some interferon responsive genes, such as major histocompatibility complex type 1 genes, and activates others, such as interleukin-12 (IL-12) gene. The DNA binding ability of ICSBP is very weak but dramatically increased by interaction with IRF 1 and IRF 2 (Bovolenta et al., 1994; Sharf et al., 1997). The interaction is mediated by the IRF association domain, which is conserved among several IRF members. ICSBP also binds to the Ets family transcription factor PU.1, which is required for development along the lymphoid and myeloid lineages of the cells.

2.7.1 Structure of ICSBP

ICSBP, a 50-kD protein, contains a DNA binding domain (DBD) which consists of a repeat of five conserved tryptophans (w) each separated by 10 to 18 amino acid residue (Harada et al, 1989). DBD is localized at N terminus and followed by IRF association domain (IAD).

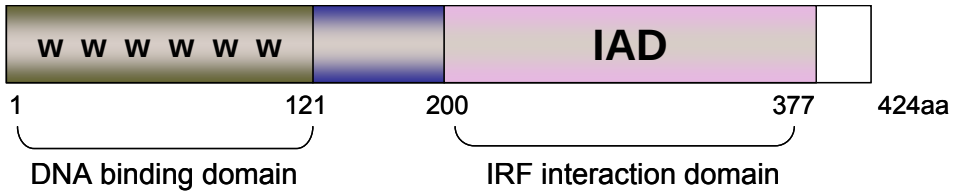

Figure 6: Structure of ICSBP protein.

Furthermore, the IAD is essential for the association of ISGF3γ/IRF-9, a non-tissue-restricted IRF member, with Stat1 and Stat2 to form the ISGF3 complex, which plays a pivotal role in IFN type I signaling (Veals et al., 1993). This suggests that IRFs-IAD modules are spatially organized like a key lock mechanism. This means, the overall structure is similar and determined by conserved residues, while non-conserved residues dictate the specificity of interactions. A point mutation within IRFs-IAD in a conserved leucine to proline (Leu^{331} in ICSBP-IAD) was able to ablate the interactions of ICSBP with IRF-1, IRF-2, PU.1, and E47, the interaction of IRF-4 with PU.1 and E47 (Meraro et al., 1999) and the interaction of ISGF3γ with Stat1 and Stat2. ICSBP is transcriptional repressor acting through the IFN stimulated response element (ISRE). ISRE and IFN consensus sequences (ICS) contain the IRF recognition sequence (IRS) AANNGAAA, to which the DBD of the IRF family binds.

2.7.2 ICSBP in chronic myeloid leukemia

ICSBP plays an important role in regulating immune responses and hematopoiesis. Interestingly, ICSBP-deficient mice manifest a CML-like syndrome characterized by enhanced proliferation of myeloid, monocytic and lymphoid lineage, suggesting role for ICSBP in regulating the proliferation and differentiation of hematopoietic progenitor cells. Transition of the chronic phase to a fatal blast crisis is a characteristic feature of human CML. Approximately 33% of ICSBP-/- and 9% of ICSBP+/- mice died by 50 weeks of age, with features indicating a transition to a blast crisis (Holtschke et al., 1996). It has been reported that *ICSBP* expression is decreased in CML patients and this reduction of *ICSBP* transcripts could be reversed by IFN-α treatment (Schmidt et al., 1998). Forced expression of ICSBP induces potent immunity against BCR-ABL induced leukemia in mice (Deng and Daley, 2001). Furthermore, ICSBP also functions as a tumor suppressor and regulator of apoptosis (Gabriele et al., 1999; Schwieger et al., 2002). ICSBP mediates its anti-oncogenic effects by down-regulating bcl-2 (one of the key anti-apoptotic cancer genes) because it is essentially required for BCR-ABL induced transformation, apoptosis, and drug resistance (Burchert et al., 2004). These data suggest that ICSBP plays an important role in the development of malignant myeloid leukemia and regulation of apoptosis.

3 AIM OF THE PROJECT

1. To identify the mechanism of disease persistence in CML patients treated with IM.

Treatment with the Abl-kinase specific inhibitor IM is very effective in chronic myeloid leukemia. However, IM presumably fails to eradicate CML stem cells leading to disease persistence and relapse after IM-discontinuation. Although causes of CML persistence under IM remain ill defined, quiescence and BCR-ABL overexpression of CML stem and progenitor cells have been suggested as underlying mechanisms. The aim of this study was to track residual disease in different bone marrow fractions of patients in MMR in order to subsequently genetically analyse individual persisting clones.

2. To identify the role of ICSBP deficiency in BCR-ABL induced transformation and emergence of resistance to IM.

Even after recent therapeutic strategies in CML, resistance and persistence of leukemic (stem) cells under TKI therapy still remains a significant clinical problem. Mutations in ABL kinase domain account for TKI-resistance in about 90% of BCR-ABL positive ALL, but only in ~30% of CML patients. Still little is known about mutation-independent mechanisms of TKI resistance and the nature of kinase mutation emergence. However ICSBP is known to induce apoptosis in BCR-ABL transformed cells. Here, we aim to elucidate whether apoptosis sensitivity, of BCR-ABL transformed cells, induced by ICSBP linked to point mutation development as a major IM resistance mechanism.

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Chemicals

Chemicals	Source
Acrylamide/Bis-acrylamide	Roth
Agar	Carl Roth
Agarose	Gibco-BRL,
Ammoniumperoxodisulfat (APS)	Sigma-Aldrich
Ampicillin	Sigma-Aldrich
β-mercaptoethanol	Carl Roth
Bovine serum albumin (BSA)	Sigma-Aldrich
Bromophenol blue	Serva
Calcium chloride	Merck
Chloroquin	Sigma-Aldrich
Complete protease inhibitor	Roche
Dimethyl sulfoxide (DMSO)	Merck
1,4-Dithiothreitol	Roth
d NTPs	Invitrogen
Ethanol	Carl Roth
Ethidium Bromide	Sigma-Aldrich

Ethylene diaminetetraacetic acid disodium salt (EDTA)	Merck
Ficoll-PagueTM PLUS	Amersham Biosciences
Formaldehyde	Carl Roth
Gene Ruler™ 100bp ladder	MBI Fermentas
Glycerol	Merck, Darmstadt
Glycine	Sigma-Aldrich
Hydrochloric acid	Merck
Hydrocortisone	Stem Cell Technology
Igepal CA-630 (indistinguishable from NP-40)	Sigma-Aldrich
IM mesylate	Novartis Pharma
Isopropanol	Carl Roth
LB Agar	Invitrogen
LB Broth Base	Invitrogen
L-Glutamine	Gibco BRL
Methanol	Merck
Milk powder	Merck
N-Ethyl-N-Nitrosurea	Sigma- Aldrich
Orthovanadate	Sigma- Aldrich
Ouabain	Sigma- Aldrich
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich
Polybrene (Hexadimethrin Bromide)	Sigma- Aldrich

Ploy-L-Lysine	Sigma- Aldrich
Propidium iodide(PI)	Sigma- Aldrich
Retronectin	Takara
Sodium Butyrate	Upstate
Sodium chloride	Sigma-Aldrich
Sodium dodecyl sulfate (SDS)	Merck
Sodium hydroxide	Merck
Sodium Orthovanadate	Sigma- Aldrich
N,N,N′,N′-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich
Tris(hydroxymethyl)aminomethane (Tris)	Roth
Tween®20	Roth

4.1.2 Cell Culture Media, cytokines and Antibiotics

Reagent	**Source**
RPMI 1640 medium (72400-021)	Gibco-BRL
IMDM medium (SH30228.01)	HyClone® HyClone
DMEM medium (41965)	Gibco-BRL
X-vivo medium	Lonza Biowittekar
MethoCult H4330	Stem Cell Technologies
MethoCult H5100	Stem Cell Technologies

Collagen Solution	Stem Cell Technologies
Ampicillin	Sigma-Aldrich
Penicillin/Streptomycin (15140-114)	Biochrom KG
Hygromycin B	Roche
Puromycin	Sigma- Aldrich
Blasticidin	Sigma- Aldrich
G418 sulphate	PAA
BSA	Stem cell technology
Fetal calf serum (FCS)	Gibco-BRL
Trypsin	Gibco-BRL
Recombinant mouse IL3	Immuno tools
Recombinant mouse IL6	Immuno tools
Recombinant mouse SCF	Immuno tools

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4.1.3 Equipment

Hardware	**Manufacturer**
Autoclave	Stiefenhofe
Agarose gel electrophoresis chambers	BioRad
Bacterial shaker	Innova, New Brunswic Scientific

Balance machine	Kobe
Electrophoresis power supply	Pharmacia Fine Chemicals
ELISA reader	Labsystems Multiskan RC
FACS SCAN	Becton Dickinson
Gel camera	PeqLab Biotechnologie GmbH
GeneAmp® PCR system	9600 Applied Bioystems,
Heating blocks	Eppendorf
PCR machine	Eppendorf
pH meter	Fisher Scientific
Ice machines	Genheimer
Inverted microscope	(DMIL) Leica, Wetzlar
Laminar hoods	Heraeus
Liquid nitrogen tank	Tec-Lab
Micro liter pipettes	Gilson
MoFlo	Dako Cytomation
OPTIMAX® film processor	PROTEC processorTechnology
Power supply units (MODE/250EX)	Gibco BRL
Semi-dry blot apparatus	BioRad, München
Sequence detection system (ABI PRISM 7700)	PE Applied-Biosystems
Water bath	Unitherm
X-ray film processor (UVT 2035)	Herolab

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4.1.4 Kits

Kit	Source
BCA Protein Assay Kit	Prerce
cDNA Synthesis Kit	Invitrogen
Gel extraction Kit	Qiagen
MACS separation Kit	Milteney Biotech
Plasmid Mini prep Kit	Qiagen
Plasmid Maxi prep Kit	Qiagen
RNA isolation Kit	Qiagen
Lineage Cell Depletion Kit, Mouse	Miltenyi Biotech
BCR-ABL Mbcr Fusion*Quant* Kit	Ipsogen

4.1.5 Miscellaneous

Cryotubes	Greiner bio-one
Disposable needles, cuvettes & syringes	Greiner bio-one
DNA and protein size markers	Fermentas
Glasswares	Schott
Nitrocellulose membrane	Amersham Life Science

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Polypropylene tubes	Greiner bio-one
Parafilm	Greiner bio-one
Pipette tips	Greiner bio-one
Röntgen film (13x18 cm, BioMax)	Kodak
Tissue culture plates	Greiner bio-one
Tissue culture flask (T-25, T-75 & T-250)	Greiner bio-one
Tissue culture dish (60 mm, 90 mm)	Greiner bio-one
Tubes (1.5 and 2 ml)	Eppendorf
Whatmann paper	Schleicher & Schuell
6,12 and 24 well cell culture plates	Cellstar

4.1.6 Cell Lines

List of the cell lines used in the study

1. 32D cells (murine mayeloid cell line)

2. M210B4 cells (murine stroma cell line)

3. K562 cells (Human erythromyeloblastoid leukemia cell line)

4. U937 cells (Human leukemic monocyte lymphoma cell line)

5. Phoneix cells (human embryonic kidney cell line)

4.1.7 Stable cell lines generated during study:

1 .32D BCR/ABL

2. 32D-MIGR1 (control vector)

2. 32D BCR/ABL- ICSBP

3. 32D BCR/ABL-Bcl2

4. 32D BCR/ABL-ICSBP-Bcl2

5. 32D-ICSBP-GFP

6. 32D-pSF91 (Control vector)

4.1.8 Plasmid and construct

MIGR1- BCR ABL (p210)

MIGR1 (control plasmid)

pSF91 –eGFP- PRE (control plasmid)

pSF91 –ICSBP –eGFP- PRE (cloned during study)

pMY-mAID-IRES-EGFP

pMY-IRES-EGFP (control plasmid)

pBABE puro-Bcl-2

pBABE puro (control plasmid)

4.1.9 PCR Primer sequence for cDNA:

h-*ICSBP*	5’ GTC CCA ACT GGA CAT TTC CG 3’
	5’CAT TCA CGC AGC CAG CAG -3’
h-*GAPDH*	5’CTC CTC CAC CTT TGA CGC TG 3’
	5’ ACC ACC CTG TTG CTG TAG CC 3’
h-*Actin*	5’CCT TCC TGG GCA TGG AGT CCT 3’
	5’AAT CTC ATC TTG TTT TCT GCG 3’
Bcr/abl	5’TTC AGA AGC TTC TCC CTG ACA TCC GT 3’
	5’GGT ACC AGG AGT GTT TCT CCA GAC TG 3’
Bcr/abl (nested)	5’GTC CAC AGC ATT CCG CTG ACC ATC AAT -3’
	5’TGT TGA CTG GCG TGA TGT AGT TGC TTG G 3’
m-*P21*	5’GCC ACA GAA TTC ATG TCC AAT CCT GGT GAT
	5’GCT CCC GTC GAC GGC ACT TCA GGG TTT TCT 3’
m-*AID*	5’AAA TGT CCG CTG GGC CAA 3’
	5’CAT CGA CTT CGT ACA AGG 3’
m-*PUMA*	5’GCC CAG CAG CAC TTA GAG TC 3’
	5’TGT CGA TGC TGC TCT TCT TG 3’
m-*MDM 2*	5’TGC AAG CAC CTC ACA GAT TC 3’
	5’ACA CAA TGT GCT GCT GCT TC 3’
m-*GAPDH*	5’CAT GGC CTT CCG TGT TCC TA 3’
	5’CCT GCT TCA CCA CCT TCT TGA 3’

m-*ICSBP*	5’AGA GGG AGA CAA AGC TGA ACC 3’
	5’TGA ATG GTG TGT GTC ATA GGC 3’
m-*MGMT*	5’AAG CTG GAA CTT GGC AGA AT 3’
	5’ACT GAG CAA CCG TAT CCC AT 3’
m-*HUS1*	5’TTT GCT TAC CAG CCT TGA AGA 3’
	5’CCC TTT AGG TTG GCT TCA ATC 3’
m-*LIG 1*	5’ACA TCT CCC CAT CAG GAT TC 3’
	5’TGT CCT CAT TCT GCT CCT CA 3’
m-*XRCC 2*	5’GAT GTG TAG CGA CTT TCG CA 3’
	5’ATC AGC AAA CAG GTT GGG TT 3’
m-*CHK 1*	5’GGG GTG GTT TAT CTT CAT GG 3’
	5’GCC AAG CCA AAG TCA GAG AT 3’
m-*OGG 1*	5’TAT CAT GGC TTC CCA AAC CT 3’
	5’TGG CAC TGG CAC GTA CAT AG 3’
m-*PARP-2*	5’AAG CTG GGA AAG GCT CAT GT 3’
	5’CTT GTT GTT GTT GAA CTG GAG A 3’
m-*MDB 4*	5’ACA GGA TGG CTC TGA AAT GC 3’
	5’CTA CTT GTG TCC GTG GGA TG 3’
m-*MPG*	5’CCG GCT AGG ACC AGA GTT TT 3’
	5’CCA CGG AGT TCT GTT CCA TC 3’
m-*TLK1*	5’AGG TCC CTG CTG AAT CAC AC 3’

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	5’ TTC TTG CCT TCT TGG GTC C 3’
m-*PMS-2*	5’ CCC TAG TGA CGC TGT GTG TG 3’
	5’ CGT GCA CTG TGA AAT GAA GC 3’
m-*POLD-3*	5’ TTC ATG TTA ACC AGG CCA AA 3’
	5’ GAC TGC CAG ACA CCA AGT AGG 3’
m-*MUTYH*	5’ AGA ACA CGT GCC CTT AGC AG 3’
	5’ TGA TAT GGG GAG ACA GAG GC 3’

4.1.10 Antibodies for western blot

cABl (24-11)	Santacruz Biotechnology
p-CrkL (3181L)	Cell Signaling
p-STAT 5 (8-5-2)	Upstat
β-Actin	Sigma
Bcl 2 (7/Bcl2)	Transduction lab
p-Akt	Cell Signaling
Akt (H-136)	Santacruz Biotechnology
ICSBP(C 19)	Santacruz Biotechnology
CrkL (C-20)	Santacruz Biotechnology

4.1.11 Antibodies for FACS analysis:

Antibodies	**Source**

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CD2 – FITC (S5.2), Human	BD Biosciences
CD3- FITC (HIT3a), Human	BD Biosciences
CD4- FITC (RPA-T4), Human	BD Biosciences
CD8 – FITC (HIT8a), Human	BD Biosciences
CD14 - FITC (MΦP9), Human	BD Biosciences
CD19 - FITC (ID-3), Human	BD Biosciences
CD20- FITC, Human	BD Biosciences
CD56, Human	BD Biosciences
GPA, Human	BD Biosciences
CD34- PE Cy 7 (8G12), Human	BD Biosciences
CD 38 PerCP-Cy5.5 (HIT2), Human	BD Biosciences
CD123 PE (9F5), Human	BD Biosciences
CD45RA- APC (HI100), Human	BD Biosciences
CD 34- PE (RAM34), Mouse	eBioscience
Sca1- Pacific blue (E13-161.7), Mouse	Biolegend
c-Kit- APC (2B8), Mouse	eBioscience
FcγR- PE-Cy (93), Mouse	eBioscience
Lineage Biotin Antibody Cocktail mouse	Miltenyi Biotech
Anti –Biotin MicroBeads	Miltenyi Biotech
CD 34 MicroBeads, human	Miltenyi Biotech

FcR Blocking Reagent	Miltenyi Biotech
Annexin V- FITC	

4.1.12 Mice

6 to 8 weeks old pathogen-free C57 Black6 mice and ICSBP knock out mice were obtained from Berlin, Germany. They were kept in the animal care facility of Institute of molecular biology and tumour research, Marburg. Institutional guidelines were followed in handling mice throughout the course of the study.

4.1.13 Patient samples

Primary CML-progenitor cells were isolated from bone marrow aspirations (30ml) during diagnostic biopsies of patients with first diagnosis (FD) of chronic phase of CML (table 1) or during routine follow up biopsies for cytogenetic analysis of patients during at least MMR (table 2). Patients were treated within multicenter CML studies in Mannheim or Marburg University Clinic and gave written informed consent to the donation. (Ethics proposal number 35/08 and 09/04, The Ethics Committee of University of Marburg)

4.1.14 Buffers and solutions

10%APS (W/V): 1g ammoniumpersulfat in 10ml destilled water.

10×Electrophoresis buffer

250mM Tris-OH,

1.9M Glycine,

2.5%SDS.

MACS buffer

2μM EDTA,

0.5%BSA in PBS.

10× Red blood cell lysis buffer

Dissolved the follow

8.02g Ammoniumchlorid,

1g potassium bicarbonate,

0.037g EDTA with H_2O to 100ml.

Radioimmuno-precipitation Assay Buffer (RIPA Buffer)

20 mM Tris pH 7.5

150 mM NaCl

1% Nonidet P-40

0.5% Sodium Deoxycholate

1 mM EDTA

0.1% SDS

Protein Loading Buffer (1X SDS gel loading buffer)

50 mM Tris-Cl (pH 6.8)

100 mM Dithiothreitol

2% SDS

0.1% Bromophenol blue

10% Glycerol

Sodium dodecyl sulphate -Polyacrylamide gels (SDS-PAGE)

Resolving Gel

Component volumes (ml) per 10 ml gel mix for 8% to 12%

H_2O 3.3 - 4.6 ml

30% acrylamide mix 2.7 ml - 4.0 ml

1.5 M Tris (pH 8.8) 2.5 ml

10% SDS 0.1 ml

10% ammonium persulfate 0.1 ml

TEMED 0.004 ml

Stacking Gel

Component volumes (ml) per 3 ml gel mix for 10% to 15%

H_2O 2.1 ml

30% acrylamide mix 0.5 ml

1.0 M Tris (pH 6.8) 0.38 ml

10% SDS 0.03 ml

10% ammonium persulfate 0.03 ml

TEMED 0.003 ml

Strip-buffer

0.1M β–Mercaptoethanol,

2% SDS and

62.5mM Tris HCL (pH 6.7).

1×TAE (pH 8.0) buffer 40mM Tris-acetate/1mM EDTA.

10× TBS buffer 200mM Tris-OH pH 7.5, 1.37M NaCl.

1× TBST buffer 1L 1×TBS and 1ml Tween 20.

Table 1 CML patient details (MMR patients)

	Patient (gender, age[§])	Euro Risk Score	Dose of IM	Years from First Diagnosis	Years on IM	Molecular Remission at the time of Sorting (IS)	Most Recent Molecular Remission (IS)
1	**(f, 59)**	**low**	**400 mg**	**1 ***	**1**	**CMR**	**CMR**
		831		**2.3 ‡**	**2.3**		
2	**(f, 39)**	**low**	**800-400 mg**	**2 † ‡**	**2**	**0.033%**	**0.017%**
		614		**3 ***	**3**	**0.0097%**	
				4 ‡	**4**	**0.019 %**	
3	**(m, 73)**	**interm**	**400 mg**	**3 †**	**3**	**CMR**	**CMR**
		911		**4 * ‡**	**4**		
4	**(m, 57)**	**interm**	**400mg +IFN**	**4***	**4**	**0.019%**	**0.0076%**
		1313					
5	**(m, 57)**	**interm**	**400 mg**	**2.4 * ‡**	**2.4**	**0.025%**	**0.011%**
		1118.3					
6	**(m, 71)**	**interm**	**800-400 mg**	**0.10 ***	**0.10**	**CMR**	**CMR**
		870.5		**1.10 ‡**	**1.10**	**CMR**	**CMR**
7	**(m, 31)**	**interm**	**400 mg**	**1 ***	**1**	**0.26 %**	**0.068%**
		1098.1					
8	**(f, 59)**	**unknown**	**400 mg**	**2.1 ***	**2.1**	**CMR**	**CMR**
9	**(m, 58)**	**interm**	**800 mg**	**2.7 ***	**2.7**	**0.0025%**	**0.023%**
		1438					
10	**(f, 41)**	**low**	**400 mg**	**1.9 † ‡**	**1.9**	**0.014%**	
		445.3		**3 ***	**3**	**0.064%**	**0.031%**
				4 ‡	**4**	**0.031%**	
11	**(f, 80)**		**400 mg**	**1 ***	**1**		

12	**(m, 53)**	**interm**	**400 mg**	**1.7** †	**1.7**	**0.24%**	**0.034%**
		1432		**2.7** * ‡	**2.7**	**0.041%**	
				3.8 ‡	**3.8**	**0.034%**	
13	**(m, 38)**	**low**	**400 mg**	**6.3** †	**5.6**	**0.047%**	**0.041%**
		369.1					
14	**(m, 56)**	**unknown**	**400 mg**	**7.4** †	**7.4**	**CMR**	**CMR**
15	**(m, 27)**	**low**	**400 mg**	**4.9** †	**4.9**	**CMR**	**CMR**
		245.2					
16	**(f, 40)**	**low**	**800-400 mg**	**1** † ‡	**1**	**0.012%**	**0.0085%**
		699.5		**2.10** ‡	**2.10**	**0.0085%**	
17	**(f, 64)**	**interm**	**800-400 mg**	**2.3** †	**2.3**	**0.025%**	**0.0097%**
		994.4					
18	**(f, 73)**	**interm**	**400 mg**	**4.5** † ‡	**4.3**	**0.049%**	**0.019%**
		1443.7					
19	**(m, 67)**	**low**	**800 mg**	**4.5**	**4.5**	**0.012%**	**0.027%**
		749					

Abbreviations: cmR, complete molecular remission; IS, international scale; conventional; m, male; f, female;

*** Analysis of bcr/abl expression in individual colony forming units, ‡ Analysis of bcr/abl expression in total sorted fractions, † Analysis of bcr/abl expression by nested PCR**

Table 2 CML-patient details (first diagnosis)

Patient (gender, age)	Risk Score	Karyotype
1 (m, 77)	**interm (1035)**	**46,XY, t(9;22)(q34;q11)**
2 (m, 69)	**interm (994.4)**	**46,XY, t(9;22)(q34;q11)**
3 (m, 69)	**high (2580)**	**46,XY, t(9;22)(q34;q11)**
4 (f , 48)	**low (203.9)**	**46,XX, t(9;22)(q34;q11)**
5 (f, 62)	**interm (1379.6)**	**46,XX, t(9;22)(q34;q11)**
6 (m, 35)	**low (471.6)**	**46,XY, t(9;22)(q34;q11)**
7 (f, 51)	**interm (917.2)**	**46,XX, t(9;22)(q34;q11)**
8 (f, 44)	**low (123.9)**	**46,XX, t(9;22)(q34;q11)**
9 (m, 38)	**low (409.4)**	**46,XY, t(9;22)(q34;q11)**

Abbreviations: m, male; f, female

4.2 METHODS

4.2.1 Isolation of bone marrow mononuclear cells (BMMNCs)

BMMNCs were isolated by means of Ficoll-Hypaque density-gradient centrifugation. Briefly, carefully added bone marrow on the top of Ficoll in the 50ml tube, centrifuged for 20 minutes at 800g, middle layer cells were carefully taken out and washed with PBS, lysed with 1× red blood cell lysis buffer for 5 minutes to remove red blood cells, again washed with PBS.

4.2.2 Enrichment of CD34 positive cells

BMMNCs resuspended in MACS buffer (0.2M EDTA, 0,25g/ml BSA) were mixed with FcR blocking reagent and CD34 magnetic micro beads and incubated on ice for 20 minutes. The mixture was loaded on pre-washed column with MACS buffer, which was entrapped in strong magnetic field. The column was then washed with MACS buffer twice. The cells were harvested by removing the column from the magnetic field. $CD34^+$ cells were washed with MACS buffer and immediately used for sorting of HSC and myeloid progenitors.

4.2.3 Separation of HSC and progenitors from human bone marrow

MACS-enriched $CD34^+$ cells from FD and MMR patients were stained with FITC-conjugated antibodies against lineage antigens including CD2 (S5.2), CD3 (HIT3a), CD4 (RPA-T4), CD8 (HIT8a), CD14 (MΦP9), CD19 (ID-3), CD20 (2H7), CD56 (NCAM 16.2) and GPA (HIR2) (BD Bioscience, Heidelberg, Germany) to gate for lin-negative (lin^-) cells. In addition, cells were also stained with PE-Cy 7 conjugated anti-CD34 (8G12), PerCP-Cy5.5 conjugated anti-CD38 (HIT2), PE conjugated anti-CD123 (9F5), APC conjugated anti- CD45-RA (HI100) antibodies (BD Bioscience, Heidelberg, Germany) to define a population

that enrich for hematopoietic stem cells (HSC) referred to as $lin^-CD34^+CD38^-$. Myeloid progenitor subpopulations were sorted from the $lin^-CD34^+CD38^+$ fraction with antibodies against CD45-RA and IL3-receptor α (IL-3Rα), which separated three distinct populations: common myeloid progenitors (CMP) as $Lin^- CD34^+CD38^-$ IL-$3R\alpha^{low}$ $CD45RA^-$, candidate granulocyte macrophage progenitors (GMP) as $Lin^-CD34^+CD38^-$ IL-$3R\alpha^{low}CD45RA^+$) and megakaryocyte erythroid progenitors (MEP) as $Lin^-CD34^+CD38^-$ IL-$3R\alpha^-$, $CD45RA^-$. Cells were analyzed and sorted as previously described by MoFlo cytometer (Wang, Y. Blood 2007)

4.2.4 Retroviral infection and generation of stable cell lines

Retroviral particles were generated by transient calcium phosphate co-transfection of phoenix eco cells with the retroviral vector plasmid Mig 210 (kind gift from Dr. W. Pear, University of Pennsylvania, Philadelphia, PA), MigR1, pSF91-ICSBP, pSF91, pBABE puro-Bcl-2, followed by harvesting of virus containing supernatant after 2 days. Transduction was carried out by spinoculation (2500 rpm) for 90 min at 32°C in presence of polybrene 4ug/ml, followed by 48 hr of incubation. Retroviral supernatant were used to transduced the 32D and mice bone marrow cells.

Green fluorescent protein positive (GFP^+) (BCR/ABL)-high and -low populations 32D cells (32D/BA-high and -low), 32D-vector, 32D-ICSBP, 32D-BA, 32D BA-ICSBP, 32D- BA-AID-GFP and 32D-BA-ICSBP-AID-GFP cells were selected by flow cytometry sorting.

To generate 32D BA-Bcl-2 and 32D BA-ICSBP-Bcl-2, 24 h after infection with pBABE puro-Bcl-2 retrovirus, cells were placed in fresh growth medium and selection with 2μg of puromycin/ml was initiated. After about 15 days, individual clones picked from 96 well plates and transferred to 24 well plate and expanded to generate cell clones stably expressing Bcl-2.

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4.2.5 Retrovirus transduction of primary cells

Femur and tibia were collected from 6 to 8 week old C57Bl/6 mice and bone marrow was harvested by flushing with syringe and 26-gauge needle. Bone marrow cells were prestimulated for 48 hr in serum free X-Vivo medium (Biowittakar, Lonza) supplemented with 10% BSA, 1%(vol/vol) penicillin/ streptomycin, 200 µM l-glutamine, 6 ng/ml recombinant murine IL-3 (Immuno tools), 10 ng/ml recombinant murine IL-6 (Immuno tools), and 50 ng/ml recombinant murine stem cell factor (SCF; Immuno tools). Subsequently, cells were resuspended in virus containing supernatant and plated on retronectin -coated plates pre-exposed to viral supernatants for 30 min to allow binding of virus particles and thereby increase the cell exposure to virus. Cells were harvested 48 hours after the virus exposure and GFP^+ cells were sorted using a MoFlo in two separate populations referred as GFP^+ (BCR/ABL)-high and –low cells.

4.2.6 Cell culture

32Dcl3, K562 and U937 was obtained from DSMZ cell culture collection (DSMZ, Braunschweig, Germany) and maintained in RPMI medium supplemented with 10% FCS and 1% penicillin/streptomycin. 32Dcl3 were additionally supplemented with IL-3 (supplied as 10% WEHI-conditioned media), GFP^+ 32D-BA high and 32D-BA low cells were generated after retrovirus transduction were maintained as 32D cells, but without supplementation of IL-3. BCR-ABL expressing GFP^+ primary cells from mice bone marrow were cultured in IMDM medium supplemented with 20% FCS, (vol/vol), 1% penicillin/ streptomycin, 6 ng/ml recombinant murine IL-3, 10 ng/ml recombinant murine IL-6, and 50 ng/ml recombinant murine stem cell factor (SCF) (Immuno tools, Friesoythe, Germany). M210B4, Phoneix Eco and PlateE cells were cultured in DMEM medium supplemented with 10% FCS, 1% penicillin/streptomycin.

4.2.7 Separation of HSC and myeloid progenitor from mice bone marrow

To isolate the Lin^- cells, bone marrow cells were stained with biotinylated lineage cocktail (militeney biotech) and incubated on ice for 20 min. Anti-biotinylated antibody was added and further incubated for 20 min. Lin^- fraction were isolated using MACS columns. Lin^- bone marrow was transduced with Mig 210 virus and after 48 hours cells were stained with PE-conjugated anti CD34, APC-conjugated anti c-Kit, pecific blue-conjugated anti Sca1, PE-Cy7-conjugated anti FcγR antibodies.

After staining, cells were analyzed and sorted by MoFlo cytometer (Dako Cytomation, Fort Collins, CO). HSC identified as $lin^-c\text{-}Kit^+Sca1^+$ (LSK cells) and myeloid progenitor were sorted from $lin^-c\text{-}Kit^+Sca1^-$ (LK cells) with antibody against FcγR and CD34 which clearly separated in three distinct populations in untransduced bone marrow. Common myeloid progenitor (CMP) were identified as $lin^-c\text{-}Kit^+Sca1^-$ $Fc\gamma R^{low}$ $CD34^+$, candidate granulocyte macrophase progenitor (GMP) were identified as $lin^-c\text{-}Kit^+Sca1^-$ $Fc\gamma R^{high}$ $CD34^+$ and MEP were identified as $lin^-c\text{-}Kit^+Sca1^-$ $Fc\gamma R^{low}$ $CD34^-$.

4.2.8 Preparation of whole protein extract from Cells

Cells were harvested and washed with ice cold PBS and then resuspended in whole cell extract buffer. Then transferred them into 1.5 ml tubes and incubated on ice for 30 min. The cell suspension was centrifuged at 13,000 rpm for 10 min at 4°C and supernatant was saved as whole cell extract in another 1.5 ml tube, which was stored for future use at -80°C. Protein concentration was determined by BSA kit according to manufacturer's protocol.

4.2.9 Polyacrylamide gel electrophoresis of proteins

The polyacrylamide gel electrophoresis (PAGE) of proteins was performed in presence of 0.1% SDS according to standard Laemmli method. Protein samples were prepared by mixing with 1X SDS-PAGE sample loading buffer and boiling for 5 min to denature the protein. After Polymerization of gel, protein samples were loaded in the wells (one well was loaded with protein marker). Electrophoresis was performed at constant voltage of 120 V until the dye reached the bottom of the gel. Following the run, gels were electro-blotted onto the nitrocellulose membrane (Amersham Biosciences).

4.2.10 Western blotting

For western blotting of proteins from gel onto nitrocellulose membrane semidried blot apparatus (BIO-RAD) was used according to instructions provided by the manufacturer. Electro-blotting was performed at a constant current of 200 milli-amperes for 1 hr. Following which the membrane was incubated in 5% nonfat milk in TBST for blocking with gentle shaking at room temperature for 2 hrs. After that, the blocking solution was discarded and after washing twice with 1X TBST the membrane was incubated with an appropriate dilution of primary antibody for another 2 hrs with gentle shaking. Thereafter, the blot was washed thrice with 1X TBST for 5 min each. After washing, the blot was incubated with horseradish peroxidase conjugated secondary antibody solution for 2 hrs. The blot was washed as described above and protein were visualized and developed with ECL developing solution according to the manufacturer's instruction.

4.2.11 Long-term culture-initiating cells (LTC-IC assay)

Irradiated M210B4 fibroblasts cells were established as feeder layer, 24 hr before adding the sorted cells, in collagen coated 24-well plate. 10,000 sorted HSC, CMP, GMP and MEP cells from CML patients were cultured on the irradiated feeder layer in MyeloCult medium (StemCell Technologies H5100) with hydrocortisone according to the manufacturer's instructions. Cultures were

maintained for 5 weeks with weekly half medium exchanges. One half of the medium and cells removed and replaced with fresh medium for five weeks. After 5 weeks of culture, floating and adherent cells were harvested using Trypsin-EDTA and washed with Myelocult medium. Cells were plated into methyl cellulose medium (StemCell Technologies H4435) and colonies derived from long-term culture-initiating cells (LTC-ICs) were counted after two weeks.

4.2.12 Colony forming cell (CFC) assays

Colony forming unit assays were performed by plating 500 sorted HSC, CMP, GMP, and MEP into a 35 mm culture dish (Cellstar, Frickenhausen, Germany) using 1ml complete methylcellulose agar (MethoCult® medium H4435, StemCell Technologies) containing all necessary cytokines and reagents for CFU growth. After 14-18 days individual CFU were picked for RNA isolation. For assessment of CFU growth of BCR-ABL or mock-transduced murine progenitors 1000 GFP-positive cells were seeded in triplicate Methocult® agar in presence of IM at 3μM concentration or solvent (as control). Emerging colonies were counted after 14 days.

4.2.13 RNA isolation

RNA was isolated from 4000 sorted bone marrow cells that had been pre-enriched before sorting for $CD34^+$ cells using the MACS methodology. Cells were directly sorted (Figure 1A) into RLT-lysis buffer of the RNeasy micro kit (Qiagen, Hilden, Germany). Alternatively one individual CFU, containing approximately 100-200 cells was picked from the CFU agar and transferred into RLT buffer. Total RNA from primary cells and cell lines was extracted using Qiagen RNeasy mini kit. 1-5 X 10^6 cells were lysed in RLT buffer containing β-mercaptoethanol and passed through QIA shredder spin columns. Ethanol was added to adjust binding conditions and sample was applied to the QIAamp spin columns. RNA was bound to the silica-gel membrane during a brief centrifugation step. Contaminants were

washed away and total RNA was eluted in RNase-free water and stored at -80°C. RNA was reverse transcribed into cDNA in a total volume 20 µl using Superscript reverse transcriptase (RT) (Qiagen, Hilden, Germany) and Random Hexamer primer (Fermentas, St. Loen-Rot, Germany) under standard condition and stored at -20°C until analyzed.

4.2.14 Nested PCR for *bcr-abl* mRNA

For qualitative assessment of bcr/abl expression, cDNA from 4000 cells were used for the first round of PCR amplification at an annealing temperature of 60°C. After 40 cycles of amplification 4µl of first stage product was subjected to an additional 40 round of PCR amplification using internal nested primers. Amplification of the β-actin housekeeping gene (annealing temperature 55°C) was performed to ensure comparable loading and cDNA quality.

4.2.15 Quantitative *bcr-abl* PCR

Quantitative PCR analysis for bcr-abl expression of bone marrow samples was performed using the IPSOGEN® kit and protocol (Luminy Biotech Enterprises, France), which quantifies bcr-abl copy numbers relative to abl copy number using a real-time TaqMan® method and an ABI Prism 7700 sequence detector (Perkin Elmer Applied Biosystems, Darmstadt, Germany). 5µl cDNA were used as template in a 25µl PCR reaction. Cycling conditions were as recommended by the manufacturer initial denaturation at 50°C 2 min, 95°C for 10min, 50 cycles of amplification at 95°C for 15 sec, annealing at 60°C for 1 min. Raw ct value obtained from sample analysis was transformed in to an absolute copy number by plasmid dilution standard curve. Ratio of bcr-abl copy number to abl copy number is expressed as the normalized copy number (NCN=bcr-abl_{CN}/ abl_{CN}).

4.2.16 *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis

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ENU was dissolved in dimethlsulfoxid (DMSO) at 50 mg/ml and stored in aliquots at –80°C. ENU was added to 32D/BA-high and 32D/BA-low cells (5×10^6 cells/ml) at a concentration of 50 µg/ml followed by culturing for 24 hours. After washing three times with medium, cells were allowed to expand over one week. 32D/BA-low and –high cells were transferred into 96-well plates at 1×10^5 cells/well in a culture volume of 200 µl and supplemented with 2µM IM. Cultures were maintained for 4 weeks, replacing half of the medium every 48 hours and IM to yield a final concentration of 2µM. Wells were observed for cell growth by visual inspection under an inverted microscope and media color change every 2 to 3 days for at least 4 weeks.

4.2.17 Apoptosis measurement

Apoptosis was measured using the Annexin V-FITC apoptosis detection kit according to manufacturer's recommendations. Briefly, 1.5×10^5 cells were collected and washed once with PBS, then incubated with 5µl FITC conjugated annexin V antibody in 195µl 1× binding buffer at room temperature for 10 minutes in dark. Unbinding annexinV antibody was removed by washing with 1×binding buffer. The cells were resuspened in 1× binding buffer and acquired by FCAScan immediatedly after stained with 20ug/ml propidium iodide (PI). PI and annexinV double negative cells were recognized as surviving, Annexin V positive and PI negative cells as early apoptosis, PI positive cells was counted as late apoptosis.

4.2.18 Cell cycle analysis

1 x 10^6 cells were harvested and washed once with PBS then resuspended in 500µl PBS. Cells were fixed by dropping 3 ml 70% cold ethanol (stored at -20°C) while vortexing and stored at 4°C for overnight. Before measurement, samples were

washed once with PBS and incubated in 1 ml PBS containing 50µg PI and 200µg RNase for 30 minutes at room temperature. Following incubation, cell cycle was analyzed by FACS.

5 RESULTS

5.1 Low BCR-ABL expression levels in hematopoietic precursor cells enable persistence of chronic myeloid leukemia under IM

5.1.1 CML persistence in primitive and proliferating precursor compartments during MMR

IM induces high rates of stable complete cytogenetic remissions (CCR) in chronic phase of CML (Druker, B.J, *N Engl J Med 2006*) (Hochhaus, A, *Leukemia 2009*). However, despite long-term IM therapy, BCR/ABL-mRNA and BCR/ABL-positive CFU remain detectable during CCR (Hughes, T.P *N Engl J Med* 2003, Bhatia, R, Blood 2003). Stem cell quiescence and BCR-ABL over expression may contribute to IM insensitivity of CML-HSC (Guastafierro, S, *Leuk Res2009).*

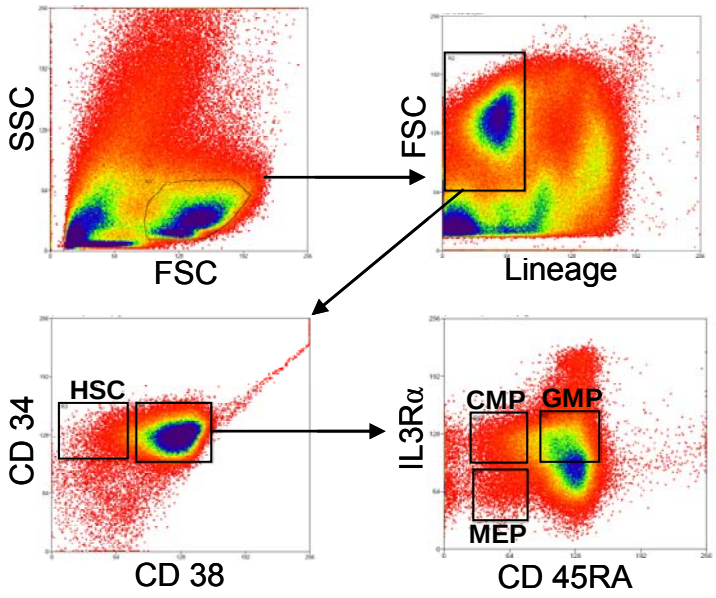

Figure 7: Sorting strategy of MACS-enriched CD34$^+$ cells. The lineage negative sub-fraction was divided into lin$^-$CD34$^+$CD38$^-$ cells, enriching for HSC and a lin$^-$CD34$^+$CD38$^+$ fraction, which was further gated according to the IL-3Rα and CD45RA staining into CMP, GMP, and MEP as indicated.

4000 cells of each population were analyzed for the presence of bcr-abl using a qualitative nested reverse transcriptase-PCR (RT-PCR) approach. As a first step to obtain insight into the nature of disease persistence in MMR patient, bone marrow of ten MMR patients was sorted into HSC, CMP, GMP, and MEP (Figure 7). BCR-ABL was detected in the HSC enriching population of seven of ten (7/10) MMR patients, but also in the CMP (6/10), GMP (10/10), and MEP (8/10) fractions of the same MMR patients (Figure 8A), indicating CML persistence of primitive and mature precursors under IM.

Persistence of mature progenitor was in contrary to the current view that IM preferentially kills dividing precursors. Therefore, we next asked, whether expansion of BCR-ABL positive progenitor compartment in presence of IM is due to acquisition of self renewal capacity. To determine this primitive and committed progenitor sorted from HSC, CMP, GMP and MEP compartment were subjected to Long term culture initiating cells assay (LTC-IC), followed by committed progenitor assay (CFU) (Eaves et al., 1992; Sutherland et al., 1990; Udomsakdi et al., 1992). After 5 weeks of LTC-IC, cells were suspended in methylcellulose medium and plated for CFU assay for detection of clonogenic cells per well. Clones that emerged after CFU assay were picked and BCR-ABL expression was analyzed by nested PCR. Similarly, CFU emerged from bone marrow of healthy donor and FD patients were analyzed for BCR-ABL expression by nested PCR. Interestingly, CFU obtained from first diagnosis patient showed a faint band after first round of PCR, whereas no BCR-ABL specific amplification was seen in MMR patient's CFU (Figure 8B). This observation led to the intriguing possibility that low BCR-ABL expressers survive IM therapy. As expected, nested PCR of CFU obtained from healthy donor was not positive for BCR-ABL transcript (Figue 8B). This assay resulted in the similar trend of CML-CFU and healthy donor CFU formation. So it cannot be used to detect self renewal capability of CML-CFU of MMR patients.

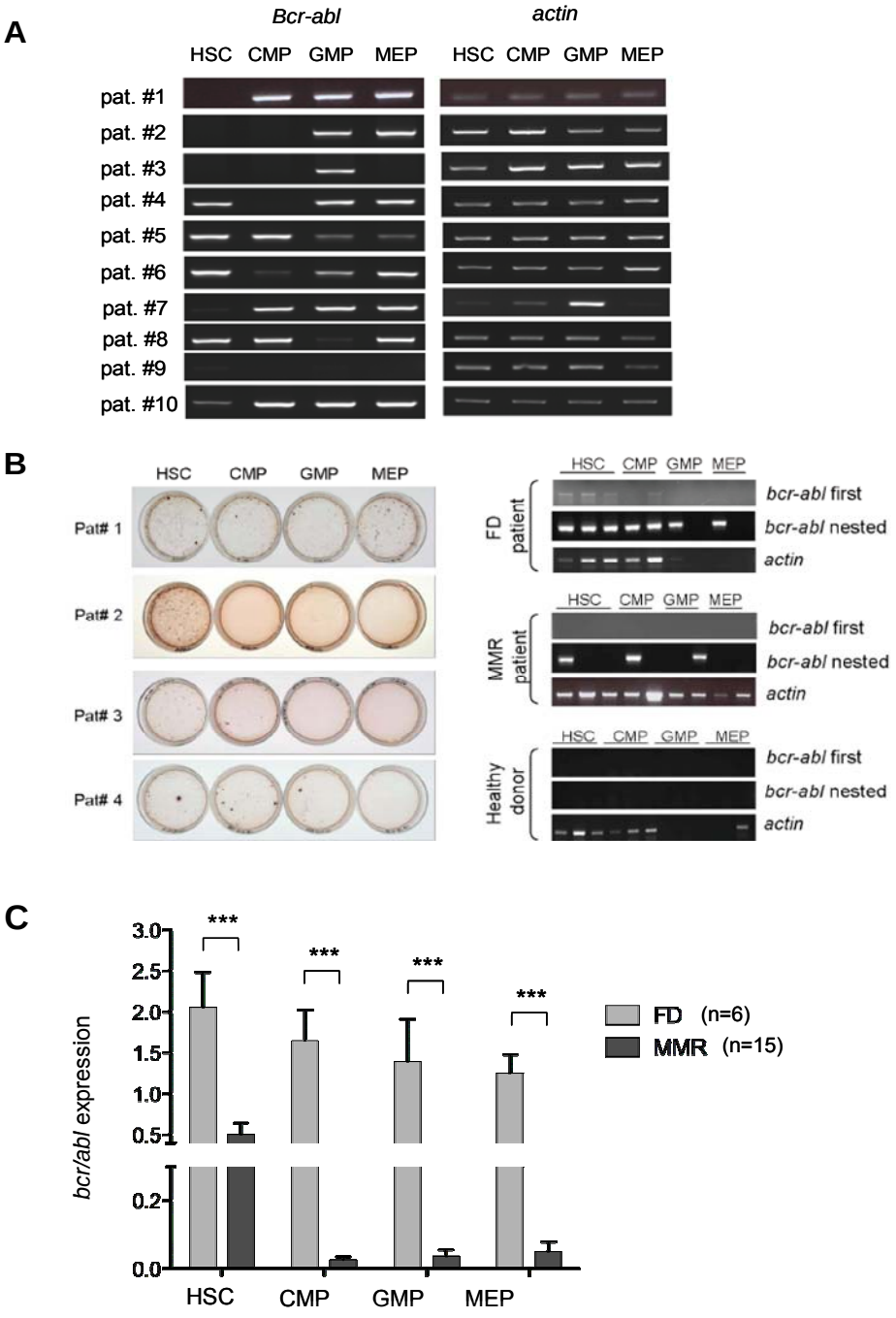

Figure 8: Detection of BCR-ABL mRNA transcripts in bone marrow subpopulations of CML-FD and MMR patient. (A) A total of 4000 cells from marrow subsets as indicated of MMR patients were analyzed for BCR-ABL expression using a qualitative nested PCR analysis; corresponding β-actin expression is shown as reference gene to demonstrate cDNA integrety. (B) 10,000 cells from each subfraction were cultured on irradiated feeder layer. After 5 weeks cells from each sub-fraction were seeded in 1 ml of methylcellulose medium with

cytokine. CFU formation in MMR patients (left). BCR-ABL expression level was determined by nested PCR in individual CFU from healthy donor, FD and MMR patient (right). (C) Quantitative real time BCR-ABL PCR normalized to ABL. Columns represent mean expression of BCR-ABL ± s.e.m. in sorted subfraction (as indicated) from CML patiens at diagnosis (CML-FD) or during MMR; *** indicates a p-value of < 0.001 according to 2way ANOVA analysis with Bonferroni adjustment for multiple comparisons.

Next a quantitative BCR-ABL PCR was performed using cDNA of primitive and committed bone marrow sub fractions of patients at CML-FD and during MMR. This revealed that BCR-ABL transcript level declined during MMR, both, in the progenitor and HSC compartments (Figure 8C). Thus, IM apparently eliminated BCR-ABL-positive cells from the mature and primitive bone marrow fractions.

5.1.2 Clearance of bone marrow from BCR-ABL positive precursors during MMR

Next we investigated in more detail, whether lower BCR-ABL expression during MMR was due to decline in the number of persisting CML precursors. In total, 251 single CFU from six FD patients and 247 CFU from 12 MMR patients, sorted from HSC, CMP, GMP, MEP compartments were analyzed by quantitative RT-PCR for bcr-abl mRNA expression. Only informative CFU yielding sufficient mRNA were considered. Out of 184 informative CFU obtained from CML FD patients, 173 were BCR-ABL-positive (94%). In contrast, only 74 of 165 CFU (44%) derived from twelve MMR patients were BCR-ABL positive (Figure 9A). The percentage of bcr-abl-positive CFU during MMR was quite varied (0%-85%, mean: 44%) (Figure 9B), but there was consistently less BCR-ABL positive CFU during MMR irrespective of whether they were isolated from committed (CMP, GMP and MEP) or primitive, HSC containing fraction (Figure 9C). This suggested that IM eliminates CML from all marrow compartments.

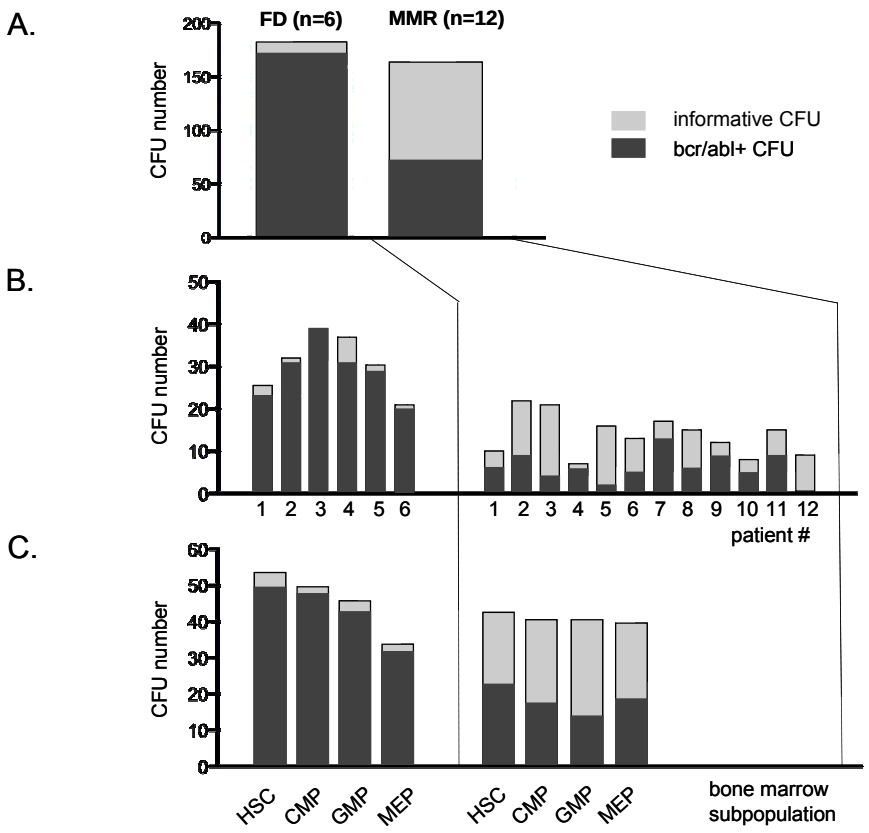

Figure 9: Frequency of BCL-ABL-negative and BCR-ABL-positive CFU at diagnosis and during MMR. BCR-ABL specific real time PCR from individual CFU: to identify the frequency of BCR-ABL-positive versus normal, that is, BCR-ABL-negative CFU at the time of CML-FD and MMR. (A) The sum of BCR-ABL-positive and normal CFU is illustrated by dark versus grey bars, respectively. (B) The amount of BCR-ABL positive and normal CFU is shown for each patient individually. (C) The number of BCR-ABL-positive and normal CFU is depicted depending on the bone marrow compartment of origin.

Moreover, it was implied that the lower BCR-ABL expression level found in the sorted sub-fractions during MMR (Figure 9C) resulted, at least in part, from a decreased number of BCR-ABL-positive CFU.

5.1.3 Reduced BCR-ABL expression in persisting CML precursors

Lower BCR-ABL expression during MMR (Figure 8B) could also be the consequence of a selective persistence of precursors with lower BCR-ABL expression.

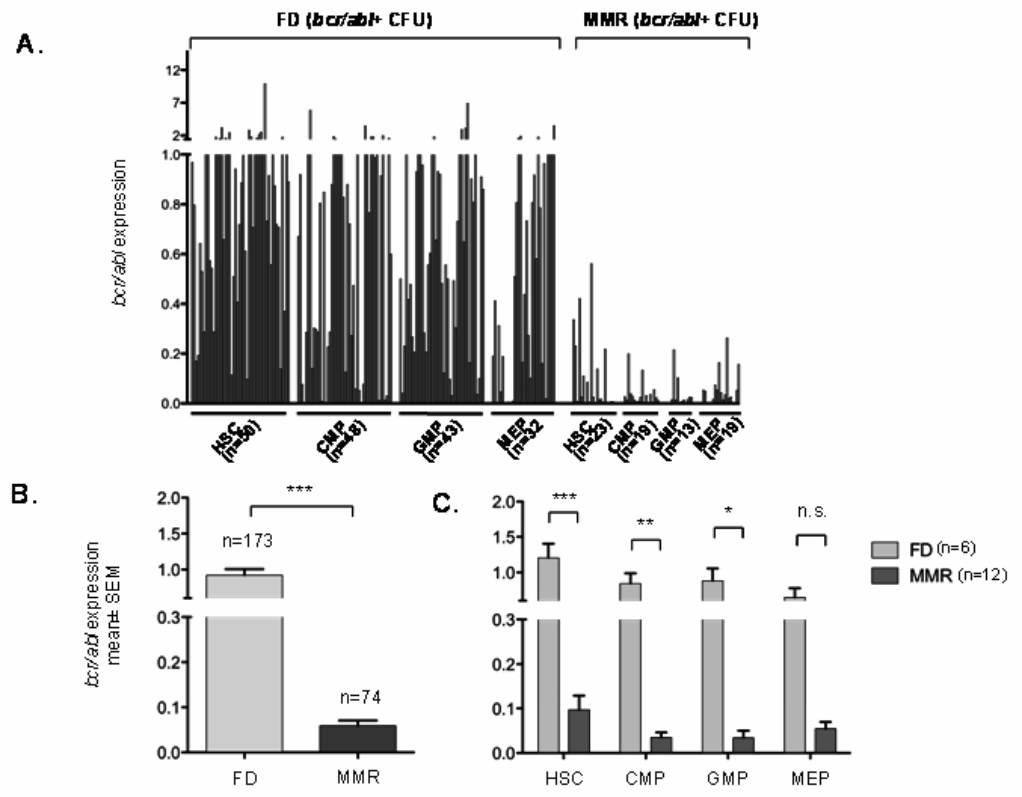

Figure 10: BCR-ABL expression analysis in individual CFU. The BCR-ABL expression levels of BCR-ABL-positive CFU from CML-FD and during MMR are shown. (**A**) Each column represents the BCR-ABL expression value of an individual CFU harvested from the indicated subfraction. (**B**) BCR-ABL expression of all analyzed BCR-ABL-positive CFU from CML-FD (grey) versus MMR (dark). Bars represent means ± s.e.m.; *** indicates high statistical significance ($p<0.0001$) according to Mann Whitney t-test. (**C**) Comparison of the BCR-ABL expression level of BCR-ABL-positive CFU from CML-FD (grey) versus MMR (dark) sorted from bone marrow compartments as indicated. Bars represent mean values ± s.e.m; *** indicates p-value <0.0001, ** $p<0.01$, and * $p<0.05$. Statistical significance was assessed using a 2way ANOVA analysis with Bonferroni adjustment for multiple comparisons.

When looking at the BCR-ABL expression level of individual CFU, we first noticed a substantial variability in BCR-ABL expression repertoire of clones from CML-FD expressed on average significantly more BCR-ABL mRNA than BCR-ABL-positive CFU from MMR patients (Figure 10B). This was true irrespective of whether comparing CML-FD and MMR CFU from the primitive HSC containing compartment, or the various committed progenitor compartments (Figure 10C).

5.1.4 Low BCR-ABL expression confers IM insensitivity of primary progenitors

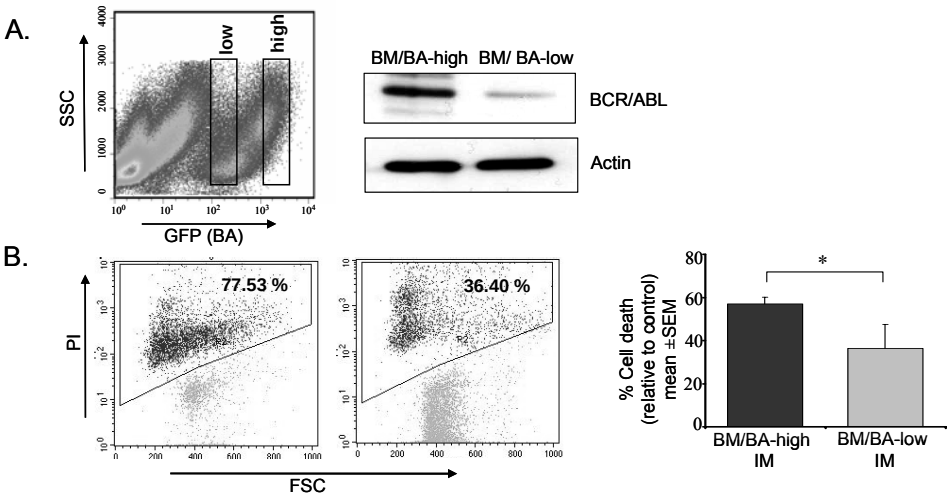

Figure 11: Effect of BCR-ABL **expression level in primary bone marrow cells on IM sensitivity.**

(A) BCR-ABL-GFP vector transduced murine BMMNC were analyzed by FACS for GFP staining intensity and sorted into GFP-high (BM/BA-high) and -low (BM/BA-low) fractions using gates as indicated (left); transduced cells were cultured for 72 hours after sorting and total protein was extracted. Western blotting was performed using anti-Abl antibodies. The membrane was reprobed using an anti-Actin antibody as a loading control. (right) (B) IM-induced apoptosis in BM/BA-high and BM/BA-low cells as assessed by propidium iodide staining. Cells were cultured for 48h with 3 μM IM and cell death was measured by PI positivity using flow cytometry. Left; representative FACS profile, gated cells are PI-positive apoptotic cells. Right; summary of results obtained from three independent experiments. Columns and error bars represent mean ± s.e.m.; * indicates $p<0.05$.

Based on the findings above, it was tempting to speculate that IM therapy preferably killed clones with higher BCR-ABL expression, resulting in the persistence of IM-insensitive disease with lower BCR-ABL expression. In order to experimentally assess, whether low BCR-ABL expression confers IM insensitivity, primary mouse bone marrow cells were transduced with a BCR-ABL construct also encoding for the expression of green fluorescence protein (GFP).

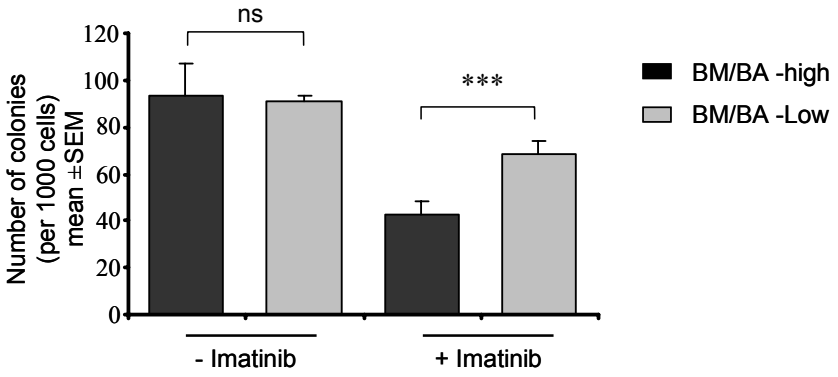

Figure 12: Effect of BCR-ABL expression level in primary bone marrow CFU. 1000 BM/BA-high and -low cells were sorted and seeded in 1ml of semisolid methylcellulose medium with and without 3µM IM, after 14 days colonies were counted. The data shown were obtained from 3 independent experiments, columns represent mean ± s.e.m., *** indicates $p<0.001$ according to Mann Whitney t-test.

The GFP-positive fraction was sorted into a GFP-high and a GFP-low expressing population to select for low and high levels of BCR/ABL (Figure 11A). The GFP staining intensity correlated with the BCR-ABL protein levels as confirmed by western blotting (Figure 11A). Sorted BCR-ABL-overexpressing bone marrow cells (BM/BA-high) and BCR-ABL-low expressing bone marrow cells (BM/BA-low) were treated with IM at a concentration of 3µM for 48h in presence of serum and growth factors. Intriguingly, BM/BA-low underwent significantly less apoptosis than BM/BA-high (Figure 11B). Moreover, IM treatment inhibited colony formation significantly more potently in BM/BA-low than in BM/BA-high ($p=0.006$). This provided evidence that lower BCR-ABL expression reduces IM sensitivity in primary bone marrow progenitors (Figure 12). There was no

significant difference in the number of colonies in BM/BA-high and BM/BA-low in the absence of IM (Figure 12).

5.1.5 BCR-ABL expression level regulate kinase mutation development

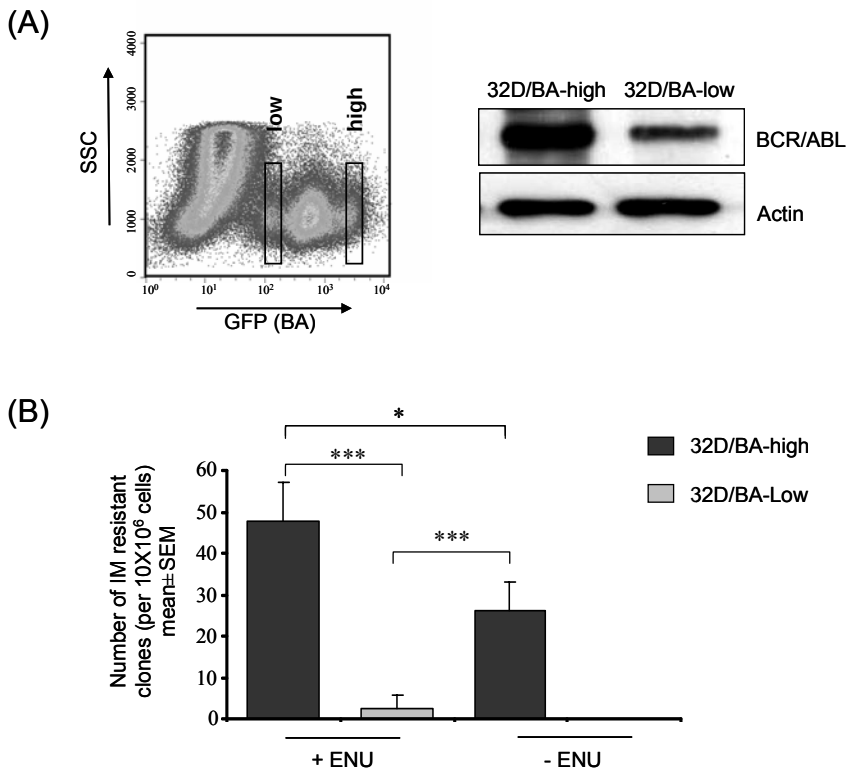

Figure 13: Effect of BCR-ABL expression and chemical mutagenesis by ENU on kinase point mutation rates. (A.) BCR/ABL-transduced 32D cells were sorted by flow cytometry on the basis of GFP staining intensity as 32D/BA-low and 32D/BA-high cells (left panel). BCR-ABL protein expression was confirmed 72 hours after sorting by western blotting using anti-Abl antibody. The blot was reprobed with anti-Actin antibody as a loading control (right panel). (B) Cell-based BCR-ABL kinase mutation assay. 32D/BA-high and 32D/BA-low cells were exposed to ENU and one week later exposed for 4 weeks to 2μM of IM. The number of outgrowing resistant clones is given relative to the total input cell number per 96 well plate. Data are representing mean± s.e.m. of 3 independent experiments; *** indicates $p<0.001$, * indicates $p<0.05$ according to 2way ANOVA analysis with Bonferroni adjustment for multiple comparisons.

BCR-ABL kinase mutations cause IM resistance (Gorre, M.E., Science, 2001; Shah, N.P.Cancer cell, 2002) and supposedly mediate persistence under IM (Chu et al., 2005). We asked, to which extend BCR-ABL expression may impact BCR-ABL kinase mutation generation. A cell-based BCR-ABL -kinase mutagenesis screen was employed, which produces BCR-ABL -kinase point mutations as the main resistance mechanism (Bradeen, H.A, Blood, 2006).

The murine myeloid 32D cell line was transduced with a BCR-ABL plasmid encoding also for GFP. Resulting 32D-BA cells were sorted according to their GFP staining intensity into putative BCR-ABL-high (32D/BA-high) and low (32D/BA-low) expressing cell populations (Figure 13A). Western blotting again confirmed that BCR-ABL expression level correlated with the GFP staining intensity (Figure 13A). 32D/BA-low and 32D/BA-high cells were exposed, respectively, to IM at 2µM and wells showing outgrowth of viable cells after 21-28 days were counted as IM resistant clones.

32D/BA-high cells generated significantly more resistant clones compared to 32D/BA-low cells. Intriguingly, the impact of chemical mutagenesis by ENU on the frequency of IM resistance development was significantly weaker than the effect of high BCR-ABL expression, because the number of emerging IM-resistant clones was a multiple fold lower in ENU-treated 32D/BA-low cells than in ENU-naïve 32D/BA-high cells ($p=0.006$) (Figure 13B). Hence, persistence of precursors with low Figure 13B expression would explain the low propensity of kinase mutation development which is clinically observed during MMR.

5.1.6 BCR-ABL expression level of CML-CFU after in-vitro treatment with IM

We also analyzed whether an in-vitro IM exposure of primary CML precursor also resulted in a selective survival of low expressing BCR-ABL CFUs. Sorted cells from FD patients were seeded in soft agar in the presence and absence of IM and resulting CFU were analyzed for BCR-ABL expression levels.

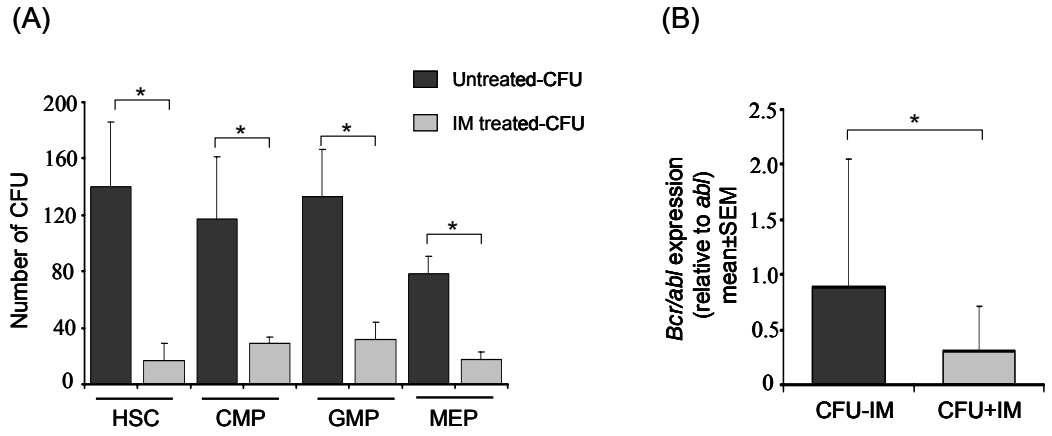

Figure 14: BCR-ABL expression level of CML-CFU after in-vitro treatment with IM. A total of 500 cells from each of the indicated marrow subsets were sorted and seeded in 1ml of semisolid methylcellulose medium with and without 3µM IM. After 14 days colonies were counted. (A) Number of CFU obtained from untreated and IM treated CFU culture. The data shown were obtained from 4 FD patients, columns represent mean ± SEM, * indicates $p<0.01$. (B) Expression of BCR-ABL was determined by quantitative PCR in untreated (dark bar) and IM treated (gray bar) single CFU. The data shown were obtained from 4 FD patients, columns represent mean ± SEM, * indicates $p=0.056$

Out of 172 informative CFU analyzed from mock treated control, 166 were *BCR-ABL* positive (95%). In contrast, 15 of 34 CFU (44%) derived from IM treated CFU were BCR-ABL positive. Although, the number of colonies was significantly reduced in IM-treated primitive and committed progenitor compared to untreated control (figure 14A). Surviving CFU after IM treatment expressed on average lower amounts of BCR-ABL than those before IM exposure. Thus, in-vitro treatment recapitulated the in vivo finding of a preferential survival of low BCR-ABL expressing progenitor cells. (figure 14B).

5.2 ICSBP deficiency in CML confers IM resistance but restricts BCR-ABL kinase point mutation development

5.2.1 *ICSBP* expression in BCR-ABL induced bone marrow

Expression of the ICSBP protein was found to be downregulated in BCR-ABL induced murine CML-like myeloproliferative disease and forced co-expression of ICSBP inhibits BCR-ABL induced CML-like myeloproliferative disease (Deng and Daley, 2001). These findings provide evidence that ICSBP can act as a tumor suppressor for BCR-ABL induced CML-like disease. It has been suggested ICSBP expression is reduced via increased promoter methylation (Ortmann et al., 2005; Tshuikina et al., 2008a; Tshuikina et al., 2008b).

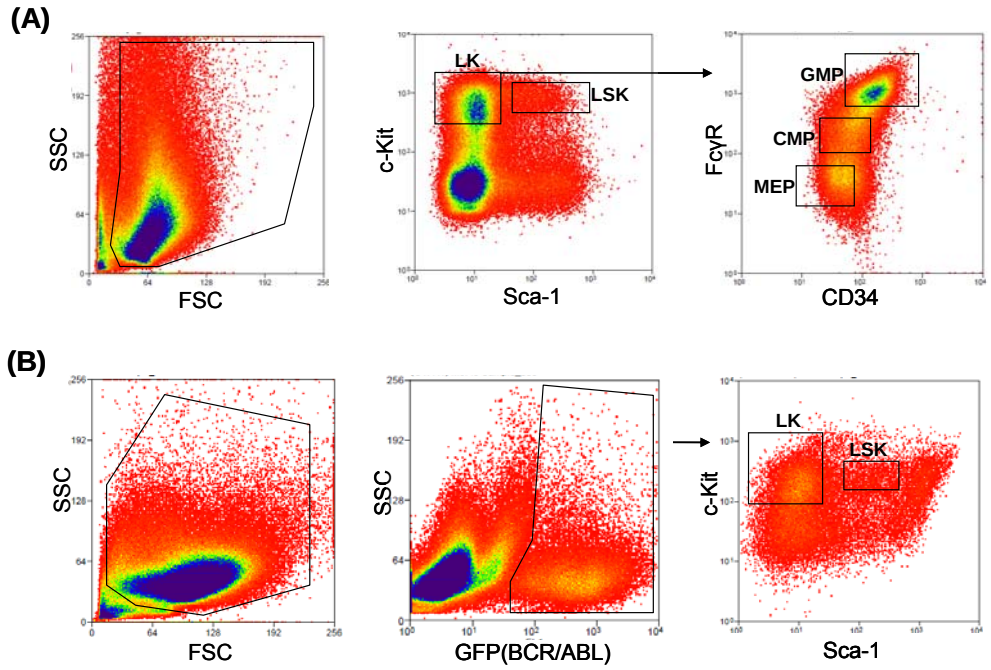

Figure 19: Sorting of stem cells and myeloid progenitor cells in mouse bone marrow: (A) Lineage negative (lin^-) cells were further stained with HSC and progenitor specific marker and sorted with MoFlo as $lin^-c\text{-}Kit^+Sca1^+$ (LKS cells) and $lin^-c\text{-}Kit^+Sca1^-$ (LK cells). LK cells were further sorted into CMP, GMP and MEP. (B) Lin^- bone marrow cells were transduced with GFP expressing Mig p210 followed by multicolor flow cytometry to sort GFP (BCR-ABL)

positive LSK and LK subsets. Viable cells were first gated for GFP^+ cells to sort the BCR-ABL positive LSK and progenitors (LK) cells.

To determine whether BCR-ABL directly suppresses ICSBP expression, lin^- cells from bone marrow of wild type mice were transduced with GFP expressing Mig p210 retroviral construct and sorted according to scheme illustrated in figure 19 (Akashi et al., 2000). Untransduced bone marrow clearly separated into LSK (HSC) and LK- fraction. The LK-fraction was further separated into CMP GMP and MEP. It was noted that BCR-ABL-transduced bone marrow produces more granulocytes–monocyte progenitors in bone marrow when compared to control untransduced bone marrow.

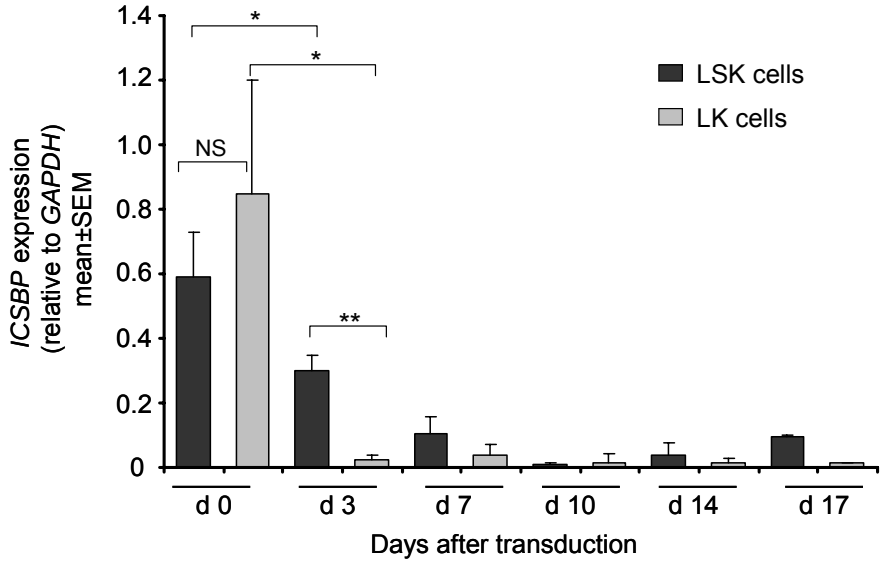

Figure 21: Expression of ICSBP in BCR-ABL transduced cells. LSK and LK cells were sorted from Mig210 trasduced and untransduced bone marrow of wild type mice and cultured for 17 days in presence of cytokines (SCF, IL-3 and IL-6). Expression of *ICSBP* was determined by real time PCR at indicated time point. Column represents mean ± SEM; *$p<0.05$ and **$p<0.005$

To examine if also the expression of *ICSBP* is changed in BCR-ABL transduced bone marrow sub-populations, expression of endogenous *ICSBP* in sorted LSK and LK cells was examined by quantitative reverse transcriptase-PCR. Sorted GFP

(BCR-ABL) positive LSK and LK cells were further expanded in presence of cytokines for 2.5 weeks and harvested on indicated time point and *ICSBP* expression was analyzed. Expression level of *ICSBP* was progressively reduced over time compared to the untransduced compartment (figure 21). Among the BCR-ABL transduced progenitors, expression levels of *ICSBP* were more down-regulated in the LK fraction than in the LSK population. Thus, in-vitro BCR-ABL transduction of primary bone marrow resulted in decreased levels of ICSBP expression. Whether this was a direct or indirect effect remained however unclear.

5.2.2 ICSBP deficiency confers IM resistance

Tyrosine kinase inhibitor IM is known to induce cell death in BCR-ABL positive cells. We next asked whether BCR-ABL dependent or independent suppression of the ICSBP also affects IM response.

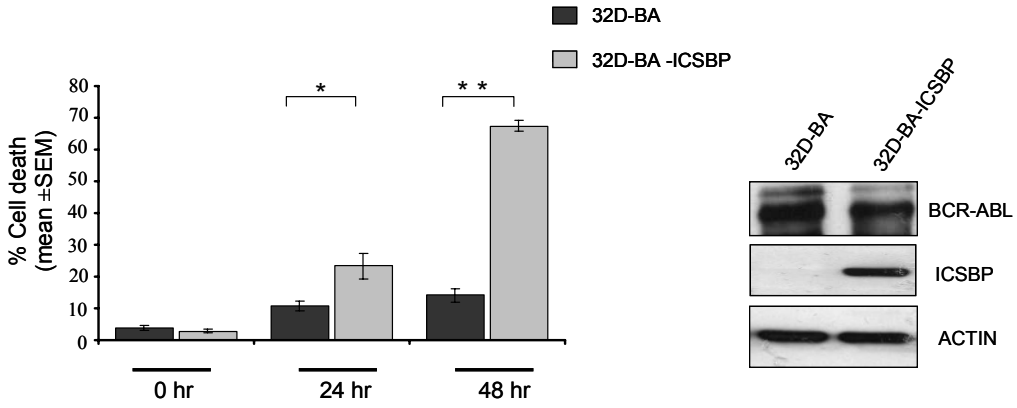

Figure 23: ICSBP deficiency confers IM resistance (Left) 32D-BA and 32D-BA-ICSBP cells were cultured for 48 hours with 1µM IM and cell death was measured using PI staining and analyzed by flow cytometry. The results shown were obtained from 3 independent experiments, column represents mean ± SEM; *p=0.001 and **p=0.0001. (Right) BCR-ABL and ICSBP protein expression in transduced cells were accessed by western blot. Cells were cultured for 72 hours after sorting and protein was extracted as described in materials and methods. Proteins were detected using anti-ABL and anti –ICSBP antibodies. Blots were reprobed with anti-actin antibody as a loading control.

In order to determine the role of ICSBP in IM induced cell death, 32D cells were stably transduced with either Mig p210 plasmid expressing BCR-ABL alone or together with another plasmid containing ICSBP to generate 32D-BA and 32D-BA-ICSBP cell lines respectively. Expression of BCR-ABL and ICSBP was checked by western blot (figure 23). IL-3 independent BCR-ABL transformed 32D-BA and 32D-BA-ICSBP cells were treated with 1μM IM. Induction of cell death occurred in time dependent manner and was measured at 24 hr and 48 hr following treatment using PI staining. 32D-BA-ICSBP cells were more sensitive to IM induced cell death than 32D-BA cells (Figure 23). The difference in the cell death was more pronounced at 48 hr compared to 24 hr.

5.2.3 Over-expression of Bcl-2 protects 32D-BA-ICSBP cells from IM induced cell death

ICSBP regulates IM induced apoptosis in BCR-ABL transformed 32D cells (Burchert et al 2004). It down-regulates one of the key anti-apoptotic gene Bcl-2, which is essentially required for BCR-ABL induced transformation, apoptosis, and drug resistance (Burchert et al 2004). Therefore, increased apoptosis in 32D-BA-ICSBP cells might be associated with reduced Bcl-2 levels. We next investigated whether enhanced expression of Bcl-2 protects against IM-induced cell death in this cell system. In order to analyze the effect of Bcl-2, 32D-BA-ICSBP cells were stably transfected with retroviral plasmid containing Bcl-2. Three puromycin-resistant clones were screened by western blot analysis for expression of Bcl-2, BCR-ABL and ICSBP (figure 24).Bcl-2-overexpressing clone (clone 3) was selected, expanded in presence of puromycin and examined for IM induced apoptosis. 32D-BA, 32D-BA-ICSBP and 32D-BA-ICSBP-Bcl2 cells were treated with 0.5, 1 and 2 μM IM for 48 hours, a concentration and time dependent effect was observed in all cells.

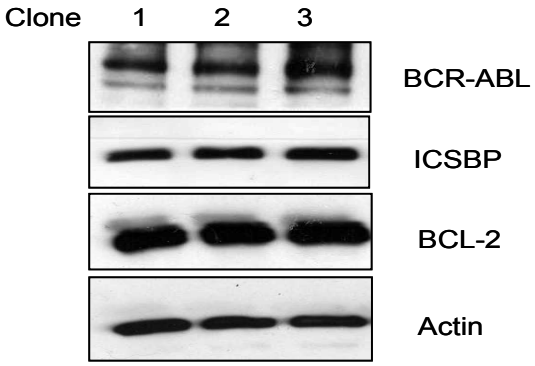

Figure 24: Selection of Bcl-2 over expressing clones. 32D BA-ICSBP cells were stably transduced with pBABE-puro-Bcl-2 plasmid and selected in presence of 2 µg/ml puomycin for 2 weeks. Several stable clones were expanded in 24 well plate and expression of BCL-2, BCR-ABL and ICSBP protein in three different clones was analyzed by western blot analysis. Blot was again reprobed with anti-Actin antibody as loading control.

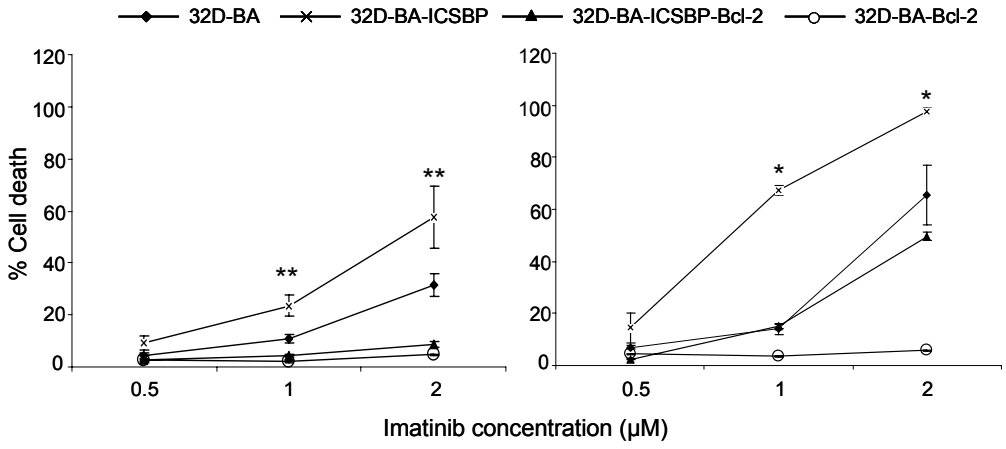

Figure 25: Effect of over-expression of Bcl2 on 32D-BA-ICSBP cells on apoptosis. After culture for 24 hours (left) and 48 hours (right) in presence of IM (0.5-2µM) cells were stained with PI and percent cell death was determined by FACS analysis. The results shown here were obtained from 3 independent experiments in which each experimental point was the mean of triplicate determination. Significance level are *P<0.0005 and **P<0.005, 32D-BA versus 32D-BA-ICSBP.

As shown in Figure 24, Bcl-2 over-expressing cells were clearly more resistant to undergo apoptosis than 32D-BA-ICSBP cells (significance level at 1μM and 2μM IM concentration was $P<0.005$ and $P<0.0005$ at 24hr and 48 hr respectively, in 32D-BA-ICSBP versus 32D-BA-ICSBP-Bcl2). Interestingly, in this cell system a complete reversal of the ICSBP induced cell death was observed through expression of Bcl-2 at 48 hr. However, 32D-BA-ICSBP-Bcl2 cells retained IM – sensitivity to some extend (approximately 50% apoptosis after 48 hrs). In contrast when only Bcl-2 was expressed in 32D-BA cells, these cells were completely IM resistant to IM at 2 μM after 48 hours (figure 25). This suggests that ICSBP induces IM sensitivity through suppression of Bcl-2.

Western blot analysis of 32D-BA, 32D-BA-ICSBP and 32D-BA-ICSBP-Bcl2 cells treated with IM resulted in similar inhibition of BCR-ABL kinase activity as shown from the inhibition of downstream target gene. Interestingly, a significantly higher inhibition of phospho-AKT was observed in 32D-BA-ICSBP and 32D-BA-ICSBP-Bcl2 cells compared to 32D-BA cells after 8 hrs treatment with IM while phosphorylation of the BCR-ABL target STAT-5 was completely inhibited by IM even at 4 hrs (Figure 26). These results indicate that IM treatment effectively inhibited BCR-ABL kinase activity, but that regulation of AKT-signaling may be BCR-ABL independent but affected by ICSBP. The AKT substrate FoxO3a, a transcription factor known to mediate growth inhibition as well as apoptosis after inhibition of AKT pathway, was also regulated by ICSBP. The basal levels of FoxO3a protein were higher in 32D-BA-ICSBP and 32D-BA-ICSBP-Bcl-2 cells compared to 32D-BA cells. This would be consistent with previous observations (Kharas et al., 2004) that increased expression of FoxO3a stimulates apoptosis. However, the exact role of ICSBP regulated FoxO3a levels needs to be further explored.

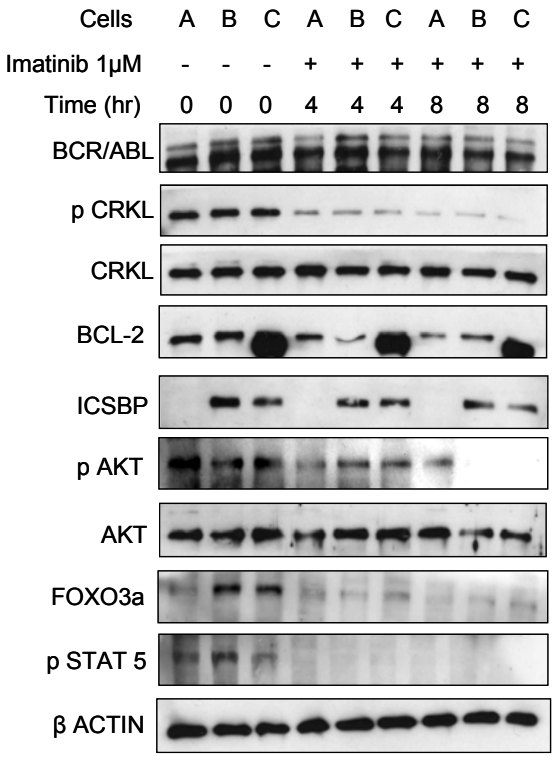

Figure 26: BCR-ABL downstream target inhibition by IM. Cells were treated with 1 µM IM for 8 hours followed by preparation of total cell extract for western blot. Cells A, B and C indicate 32D-BA, 32D-BA-ICSBP and 32D-BA-ICSBP-Bcl-2 respectively. Expression of phospho-AKT, AKT, phospho-CRKL, CRKL, phospho-STAT-5, BCL-2, ICSBP, BCR-ABL was analyzed at indicated time point. Blot was again re-probed with anti-Actin antibody as loading control.

5.2.4 ICSBP deficiency restricts BCR-ABL kinase point mutation development

BCR-ABL tyrosine kinase mutation represents a common and the best understood mechanism of IM resistance in BCR-ABL positive leukemia treated with IM. We here ask whether an increased apoptosis response in the presence of ICSBP may regulate kinase mutation development. The rate of IM-induced BCR-ABL kinase mutations was quantitated in presence and absence of ICSBP using an established TKI mutagenesis screen (von Bubnoff et al., 2006; von Bubnoff et al., 2005). This screen produces resistant clones almost exclusively via mutation induction in the BCR-ABL kinase domain.

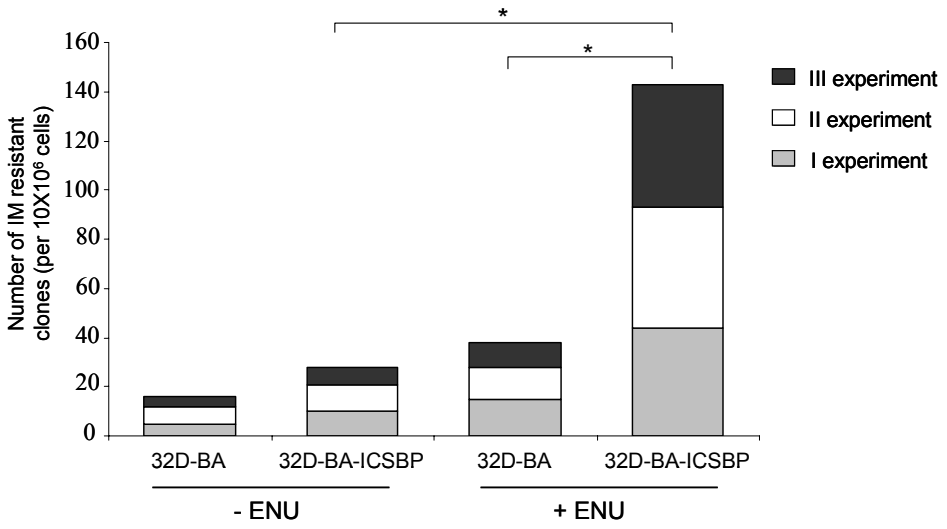

Figure 28: Role of ICSBP expression and chemical mutagenesis by ENU on kinase point mutation rates. Cell-based BCR-ABL kinase mutation assay. 32D-BA and 32D-BA-ICSBP cells were exposed to ENU and one week later exposed for 4 weeks to 2μM of IM. The number of outgrowing resistant clones is given relative to the total input cell number per 96 well plate. Data are representing mean± s.e.m. of 3 independent experiments; * indicates $p<0.0001$.

32D-BA and 32D-BA-ICSBP cells were exposed with the randomly acting mutagen, ENU (N-ethyl-N-nitrosourea) followed by IM treatment at 2μM concentration and wells showing outgrowth of viable cells after 21-28 days were counted as IM resistant clones. Unexpectedly, significantly more IM-resistant colonies emerged in 32D-BA-ICSBP cells as compared to 32D-BA cells, but only if cells were treated prior to IM-exposure with ENU (Figure 28). This suggests that the absence of ICSBP expression limits the development of kinase point mutations. Indeed, IM resistant CML patients show much less frequently kinase point mutation than IM resistant Ph^+ALL patients.

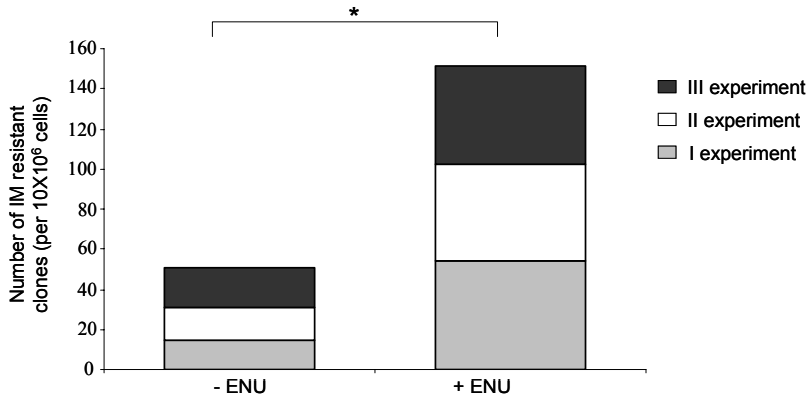

Figure 29: Effect of Bcl-2 over expression and chemical mutagenesis by ENU on kinase point mutation rates. Cell-based BCR-ABL kinase point mutation assay. 32D-BA-ICSBP-Bcl-2 cells were exposed to ENU and one week later exposed for 4 weeks to 2μM of IM. The number of outgrowing resistant clones is given relative to the total input cell number per 96 well plate. Data are representing mean± s.e.m. of 3 independent experiments; * indicates $p<0.001$,

Since ICSBP regulated apoptosis response to IM, we asked whether the increased rate of point mutations in presence of ICSBP is due to increased apoptosis sensitivity. Such a link would be novel, as little is known about the nature of kinase mutaions. We therefore over-expressed the anti-apoptotic Bcl2 gene and asked whether this prevented the increased mutagenesis rate in 32D-BAI-Bcl2 and 32D-BA-Bcl2 cells. Although over-expression of Bcl-2 could restore the apoptosis induced by IM in 32D-BA-ICSBP cells, it failed to prevent the increased rate of kinase point mutations development (figure 29).

5.2.5 PCR array targeting mouse DNA damage signaling pathway

To explore how ICSBP controls ENU induced point mutagenesis in BCR-ABL transduced cells, a DNA damage signaling pathway specific PCR Array was performed. The expression of 84 genes involved in DNA damage signaling pathways were analyzed in 32D-BA and 32D-BA-ICSBP cells treated with 50μg/ml ENU for 6 hrs. The genes targeted were associated with the ATR/ATM signaling network and transcriptional targets of DNA damage response. Out of 84

genes 52 genes were found to be down-regulated and 8 genes were up-regulated in 32D-BA-ICSBP cells as displayed in 3D profile picture.

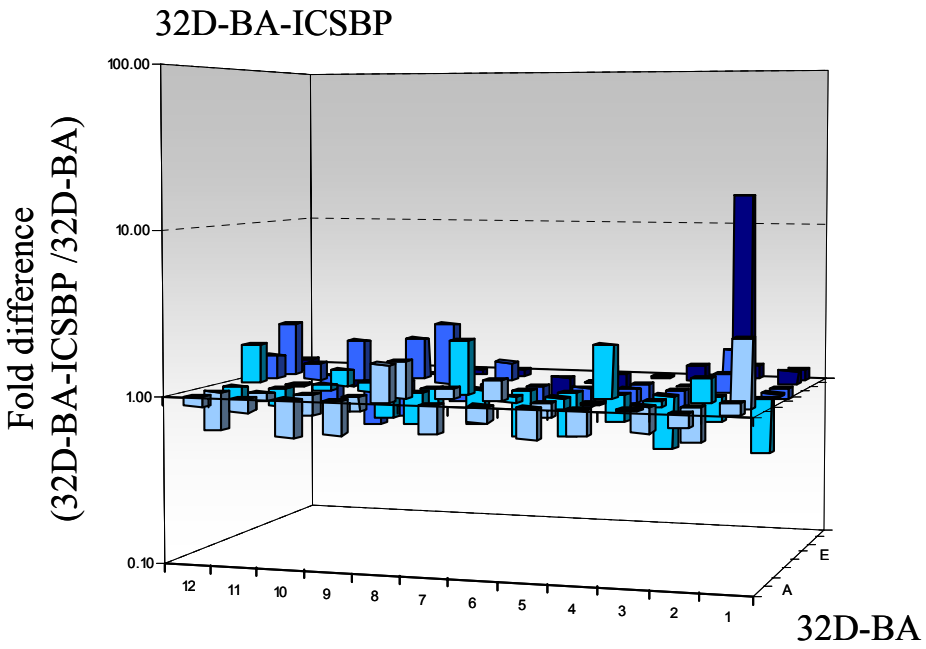

Figure30: PCR array targeting mouse DNA damage signaling pathway. Graphical display of comparative gene profiling data, shown as 3D profiler picture. Each column represents ct value of individual gene relative to housekeeping gene.

To confirm the data obtained from the PCR array, 32D-vector, 32D-ICSBP, 32D-BA and 32D-BA-ICSBP cells were treated with 50µg/ml ENU for 6 hrs and quantitative RT-PCR of selected candidate genes from PCR array was performed. Expression of 13 DNA repair pathway genes which include cell cycle related gene (*chek1, hus1* and *tlk1*), base excision repair gene (*parp2, mbd4, mpg, mutyh* and *ogg1*), damaged DNA binding gene (*xrcc2*), mismatch repair gene (*pms2* and *pold3*), apoptosis and other gene related to DNA repair (*mbd4* and *mgmt*) were analyzed by quantitative RT-PCR (figure 31).

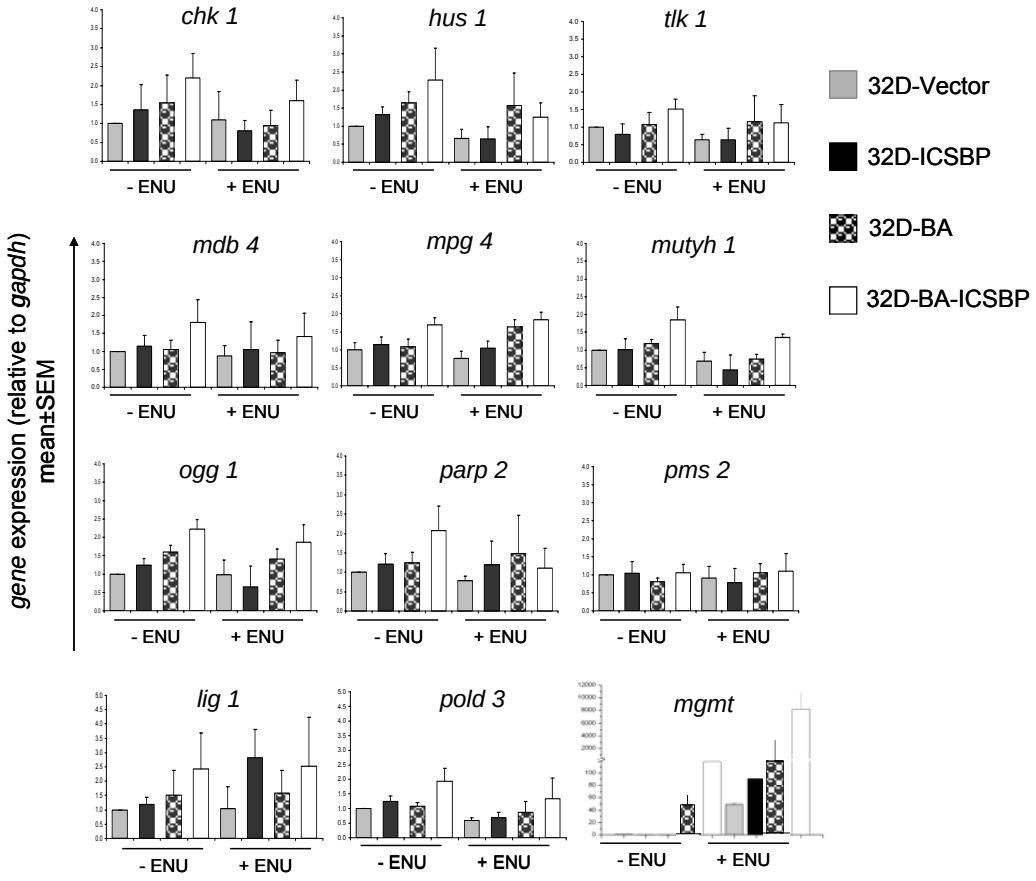

Figure 31: 32D-vector, 32D-ICSBP, 32D-BA and 32D-BA-ICSBP cells were treated with 50µg/ml ENU for 6 hours. Expression of indicated DNA repair gene was quantitated. Each column represents gene expression relative to *gapdh* (mean±s.e.m.). Results shown here were obtained from 3 independent experiments.

Only the expression of apoptosis and DNA repair related gene *mgmt* was significantly up-regulated in 32D-ICSBP cells compared to 32D-vector after treatment with ENU. Expression of mgmt was higher in 32D-BA-ICSBP compared to 32D-BA cells; expression was further increased after treatment with ENU.

6 DISCUSSION

Chronic myeloid leukemia (CML) is a clonal disorder of hematopoietic stem cells, caused by the aberrant expression of the BCR-ABL fusion protein (Ben-Nun, 1986; Rowley, 1973; Rudkin et al., 1964). IM mesylate is a specific inhibitor of BCR-ABL and induces high rates of stable complete cytogenetic remissions (CCR) in chronic phase of CML (Druker et al., 2006; Hochhaus et al., 2009). However, despite long-term IM therapy, BCR-ABL-mRNA and BCR/ABL-positive CFU remain detectable during CCR (Bhatia et al., 2003; Druker et al., 2006; Hughes et al., 2003). CML persistence supposedly results from an inherent insensitivity to IM of the CML stem and progenitor cells (Graham et al., 2002; Jorgensen et al., 2007).

A large body of evidence suggests that BCR-ABL overexpression in primitive (lin^- $CD34^+CD38^-$), and mature ($CD34^+CD38^+$) progenitors contributes to the failure of IM to eradicate CML (Copland et al., 2006; Jamieson et al., 2004; Jiang et al., 2007a; Jiang et al., 2007b). However, the BCR-ABL expression level in persisting clones, and the impact of long term IM therapy on eradication of CML in different bone marrow compartments are still largely unknown.

Abe et al. recently reported that residual disease accumulates in the lin-$CD34^+CD38^-$ fraction (Abe et al., 2008). When comparing BCR-ABL transcript level in whole marrow compartments from FD and MMR patients, we also noticed that the BCR-ABL expression goes down in the primitive (and mature) fraction, but also observed that residual disease remains detectable in the committed populations. We concluded from this, that IM is efficacious in all bone marrow compartments - independently from the proliferation status. In line with this, increasing clinical evidence suggests that long-term exposure to IM may have sustained effects on the viability of bone marrow cells including CML stem cells. For example, a significant proportion (41%) of CMR patients remained progression free at the molecular level after IM discontinuation (Mahon et al.,2010).

We specifically addressed the question, whether BCR-ABL overexpression may support long-term survival of CML clones under IM therapy. Intriguingly, we found that persisting primitive and mature BCR-ABL-positive clones (CFU) isolated from MMR patients regularly expressed significantly less BCR-ABL than FD CML CFU. This suggested that eradication of BCR-ABL -overexpressing clones present at FD could be an important requirement for the establishment of a MMR, which has an excellent long-term prognosis (Druker et al., 2006; Hughes et al., 2003; Press et al., 2006).

In turn, if IM fails to eliminate these, supposedly “dangerous” BCR-ABL -high expresser clones, it would be tempting to speculate that the odds for evolution of secondary IM resistance, emerging from these clones, will increase. Indeed, patients treated in progressed phases of CML overexpress BCR-ABL (Elmaagacli et al., 2000; Gaiger et al., 1995; Guo et al., 1991) and regularly encounter IM resistance (Druker et al., 2001; Moravcova et al., 2004; Ottmann et al., 2002). There is also solid evidence for a direct link between BCR-ABL expression level, genetic instability and IM resistance (Koptyra et al., 2008; Koptyra et al., 2006; Sallmyr et al., 2008; Stoklosa et al., 2008). Using an immortalized, BCR-ABL-dependent cell line model resembling blast crisis cells, we also provided such evidence by showing that BCR-ABL overexpression catalyzes mutagenesis and IM resistance development to a greater extent than chemical mutagenesis by ENU (Figure 5B).

The mechanism, why exactly low BCR-ABL expression level may protect from IM-induced apoptosis is not entirely clear, but could be related to induction of weaker oncogenic dependence by less BCR-ABL. Modi et al. published previously very similar results to ours. They transduced normal primary human progenitor cells engineered to express low and high BCR-ABL levels and found a diminished IM sensitivity in case of lower BCR-ABL expression .

Importantly, these findings do not contradict previous evidence that BCR-ABL overexpression may cause IM resistance (Hochhaus et al., 2002; le Coutre et al., 2000). However, it must be acknowledged that the cellular context of BCR-ABL

expression might decisively control the biological effects of the oncoprotein during transformation, progression and drug resistance.

In summary, our study supports the idea that achieving a MMR is associated with an eradication of CML precursors cells both, from the primitive and mature bone marrow fractions. It is also suggested that stable long-term persistence under IM is characterized by a selective survival of low BCR-ABL expressing precursors, which are less IM-sensitive, and genetically more stable. This would explain the low progression rates seen during MMR. Although therapeutic strategies targeting BCR-ABL-independent mechanisms of persistence may be rational (Burchert et al., ; Dierks et al., 2008; Gregory et al.), it is also possible that long-term BCR-ABL kinase inhibition per se could prevent progression and even enable cure.

Cure depends on the ability of IM to eradicate CML-stem cells. Our group has previously reported that ICSBP potently enhances the sensitivity of BCR-ABL transformed 32D cells to undergo apoptosis. ICSBP expression is lacking in CML by mechanism that are not entirely clear. It was reasonable to address to which extend and by which mechanism ICSBP expression modulates IM- sensitivity. In particular we asked whether apoptosis promoting effect of ICSBP controls the rate of kinase point mutation induction as the most important IM-resistance mechanism currently known. We stably tranduced 32D-BA and 32D-BA-ICSBP cells with Bcl2 to decrease the sensitivity of cells to undergo apoptosis in presence of IM (figure25).

However, whether apoptosis sensitivity can also regulate drug resistance development is not known. ICSBP itself regulated apoptosis sensitivity, but has also been shown to modulate somatic hypermutation (SHM) and class switch recombination (CSR) (Zhou et al., 2009) in mature B-cells, where it is overexpressed. Saberwal et al. have recently shown that ICSBP activates promoter cis element of Fanconi F (FANCF) gene which participates in repair of cross linked DNA (Saberwal et al., 2009). Thus ICSBP may regulate IM resistance via two opposing effects: promotion of apoptosis, and also hyper-mutagenesis. This

would also mean, cells expressing ICSBP can better tolerate the physiological DNA damage, which normally triggers apoptosis.

To test this hypothesis and to understand the role of ICSBP in development of kinase domain resistance, a cell based assay that uses ENU mutagenesis prior to selection of resistant clones was performed (Bradeen et al., 2006). ENU is a direct-acting alkylating agent that produces similar ratios of well-characterized ethyl adducts in DNA in cultured mammalian cells. Several O-ethyl-adducts, including O^6-ethylguanine, O^4-ethylthymine, and O^2-ethylthymine, have been shown to direct mispairing of bases during DNA replication *in vitro* (Cosentino and Heddle, 2000). Since 32D BA-ICSBP cells are more sensitive to IM induced cell death compared to 32D-BA cells, a lower mutation rate could be expected in 32D-BA-ICSBP cells. The contrary was the case, significantly higher IM resistance rate was observed in 32D-BA-ICSBP cells when compared to 32D-BA cells. Next we wanted to know whether over-expression of Bcl2 can alter increased mutation rate of BCR-ABL transformed cells. Although, overxepression of Bcl-2 in 32D-BA-ICSBP cells revert the IM induced cell death but failed to change the mutation rate in this cell system. Thus, lack of ICSBP expression in CML not only confers IM drug resistance, but also limits the frequency of point mutation development in a myeloid cell context. This would explain the lower rates of point mutations found in IM-resistant CML as opposed to BCR-ABL positive, IM-resistant ALL. In this study we also examined the relationship between ICSBP and mutagenesis in BCR-ABL transformed cells by PCR array. From this array, *mgmt* could be a potential target of ICSBP to explain the increased mutation development but further detailed investigation is required to unravel the mechanism.

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Ashu Kuamri

9 CURRICULUM VITAE

Ashu Kumari

Klinik für Hämatologie/Onkologie und Immunologie,
Universitätsklinikum Gießen und Marburg GmbH,
Standort Marburg,
Baldingerstraße,
D-35043 Marburg
Germany

Telephone (+49) 06421 – 5865112

Telefax (+49) 06421 – 5863005

E-Mail: kumaria@staff.uni-marburg.de

Education:

Masters of Science (Biotechnology) *(First Class- 74.25 %)* 2002- 2004

From Center for Biotechnology, University of Allahabad, Allahabad

Bachelor of Science (Botany, Chemistry) *(First Class- 67.9 %)* 1997- 2000

From University of Allahabad, Allahabad

Awards and Honor:

Scholarship by the Department of Biotechnology (DBT), Government of India. (Sep 2005-March 2007).

Selected for Shyma Prasad Mukhrjee Fellowship (SPM) test 2005, for top 20% of NET qualified candidates of the preceding year by Human Resource Development Group-Council of Scientific & Industrial Research (HRD-CSIR).

Qualified "National Eligibility Test for Junior Research Fellowship (NET-CSIR- JRF)", June .2004.

Qualified Graduate Aptitude Test in Engineering (GATE) 2004 conducted by Indian Institute of Technology (IIT), Delhi in Life Sciences.

Qualified "National Eligibility Test for Junior Research Fellowship (NET-CSIR- JRF)", Dec.2003.

Scholarship by the Department of Biotechnology (DBT), Government of India for Master of Science in Biotechnology (2002-2004).

Publications and presentation:

Ashu Kumari, Cornelia Brendel, Andreas Hochhaus, Andreas Neubauer, and Andreas Burchert. Low BCR-ABL expression levels in hematopoietic precursor cells enable persistence of chronic myeloid leukemia under imatinib. (Revision under ***Blood*** journal)

Ashu Kumari, Cornelia Brendel, Thorsten Volkmann, Sonja Tajstra, Andreas Neubauer, and Andreas Burchert. Persisting chronic myeloid leukemia stem and progenitor cells from patients in major molecular remission under imatinb are characterized by low bcr/abl expression. Blood (ASH Annual Meeting Abstracts), Dec 2009; 2207

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Declaration

I hereby declare that the submitted dissertation was completed by me and none other and I have not used any sources or materials other than those enclosed. Moreover, I declare that the following dissertation has not been submitted further in this form and has not been used for obtaining any other equivalent qualification in any other organization. Additionally, other than this degree I have not applied or will not attempt to apply for any other degree, title or qualification in relation to this work.

Ashu Kumari

Date

Marburg

Ehrenwötliche Erkläung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin Marburg zur Promotionsprüfung eingereichte Arbeit mit dem Titel“Elucidation of mechanism of disease resistance and persistance in chronic myeloid leukemia“ in der Klinik für Hämatologie/Onkologie/Immunologie unter Leitung von Prof Dr. Andreas Neubauer mit Unterstützung durch Prof Dr. Andreas Burchert ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation aufgeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- oder ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht, noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt.

Ashu Kumari

Date

Marburg